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Department of Biochemistry

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# Genetic and molecular dissection of the role of dissolution and resolution in the maintenance of genomic stability

**Enrico Tenaglia**

**Graduate in Molecular Biosciences**

Thesis director: **Dr. Óscar Fernández-Capetillo Ruiz**

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El **Dr. Óscar Fernández-Capetillo Ruiz**, Investigador principal del Grupo de Inestabilidad Genómica, Programa de Oncología Molecular, Centro Nacional de Investigaciones Oncológicas (CNIO)

CERTIFICA

Que **D. Enrico Tenaglia,** Licenciado en Ciencias Bio-moleculares por la Universidad de Turín - Italia, ha llevado a cabo bajo mi dirección el trabajo experimental contenido en la Tesis Doctoral "**Genetic and molecular dissection of the role of dissolution and resolution in the maintenance of genomic stability**" y reúne los requisitos necesarios para optar al título de Doctor en Ciencias.

El trabajo realizado cuenta con el visto bueno de la **Dra. Ana Aranda Iriarte**, profesora de investigación en el Instituto de Investigaciones Biológicas "Alberto Sols", que ejerce como Tutora Académica.

La Tesis será defendida próximamente en la Universidad Autónoma de Madrid ante el tribunal designado a tal efecto.

Fdo.

**Óscar Fernández-Capetillo Ruiz**
Director de tesis

Fdo.

**Ana Aranda Iriarte**
Tutora Académica

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*L'attività scientifica è materiata per grandissima parte di sforzo fantastico; chi è incapace di costruire ipotesi non sarà mai scienziato.*

*Antonio Gramsci*

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# Abstract/Resumen

## Abstract

DNA is constantly under the assault of many kinds of damage, both of exogenous and endogenous origin. The very replication of DNA molecules is a major source of this second kind of injury, for its impairment can generate a panel of toxic intermediates, where both sister chromatids are linked through DNA linkages. These structures are constantly monitored and cleared off in cells by two independent pathways, denominated dissolution and resolution. While the latter is catalyzed by structure-specific nucleases including MUS81, GEN1 and SLX1/SLX4, dissolution is known to be operated through the coordinated action of the helicase BLM and the topoisomerase TOPIIIα. A wealth of data from lower eukaryotes suggest that a complex known as SMC5/6, related to condensin and cohesin, might also be involved in dissolution – yet its contribution along this pathway in mammals is largely uncharacterized. We here used mouse models to explore a potential role of the SMC5/6 complex in dissolution in mammals. For this purpose, we employed a recently published *Smc6* mutant allele, as well as a new allele that carries a mutation on *Nsmce2*, a SUMO ligase which is an essential functional component of the SMC5/6 complex.

Our data show that the *Nsmce2*$^{SUMOdead}$ allele generated is indeed severely compromised in its SUMO ligase activity. Surprisingly, whereas some signs of genomic instability can be detected in *Nsmce2* mutants *in vitro*, our results suggests that NSMCE2-dependent SUMOylation is largely dispensable for fitness *in vivo*. In addition, we find a severe synthetic lethality between the resolution resolvase MUS81, and the *Smc6*-impairing mutation we employed. These results help to substantiate a role for SMC5/6 in dissolution in mammals, and to dimension the role that SUMOylation might play within the SMC5/6 complex. Besides dissolution, this work explored two aspects of the resolution pathway - namely, a potential role for resolution nucleases on the phenomenon of “chromosome pulverization”, and the implementation of cell-systems where the activity of these nucleases can be unleashed at will.

Collectively, my PhD tried to explore the concepts of resolution and dissolution of joint DNA molecules, something that has mostly been investigated using yeast as a model system.

## Resumen

El ADN está constantemente sometido al asalto de muchos tipos de agentes dañinos, tanto de origen exógeno como endógeno. La replicación del genoma es una fuente importante de este segundo tipo de lesión, por su capacidad de generar una serie de productos intermedios tóxicos, formados por cromátidas unidas a través del ADN. Estas estructuras son constantemente eliminadas en las células a través de dos mecanismos independientes conocidos como disolución y resolución. Mientras este último es catalizado por nucleasas estructura-específicas, denominadas MUS81, GEN1 y SLX1/SLX4, la disolución opera a través de la acción coordinada de la helicasa BLM y de la topoisomerasa TOPIIIα. Datos recientes en eucariotas inferiores sugieren que el complejo denominado SMC5/6, relacionado estructuralmente con condensina y cohesina, juega un papel fundamental en la disolución. Sin embargo, su contribución en metazoos no se ha caracterizado en detalle todavía. Para este trabajo, con el fin de explorar un posible papel del complejo SMC5/6 en mediar la disolución en mamíferos, desarrollamos y empleamos modelos de ratón mutante por SMC6, así como un nuevo alelo inactivante la actividad SUMO ligasa de NSMCE2, un componente esencial del complejo SMC5/6. Nuestros datos demuestran la efectiva abrogación functional del alelo NSMCE2$^{SUMOdead}$ . Sorprendentemente, mientras encontramos signos de inestabilidad genómica en mutantes de *Nsmce2* in vitro, los resultados expuestos sugieren que la SUMOylación mediada por NSMCE2 es prescindible para la supervivencia in vivo. Además, pudimos remarcar una severa letalidad sintética entre la mutación de la endonucleasa MUS81 y la mutación de SMC6 que empleamos. Estos resultados corroboran el papel de SMC5/6 en la disolución de los mamíferos, redimensionando la importancia de la SUMOylación operada por NSMCE2 en este contexto. Durante este trabajo caracterizamos también la resolución y su posible papel en el fenómeno de la "pulverización de los cromosomas". En conclusión, diseñamos un sistema artificial para implementar la actividad de estas nucleasas en células.

Colectivamente, durante mi doctorado intentamos caracterizar genéticamente, y a nivel molecular, la disolución y la resolución de las moléculas de ADN conjuntas, fenómenos que, a fecha de hoy, se han caracterizado principalmente en levadura.

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## Abbreviations

| | |
|---|---|
| **AOM** | *azoxymethane* |
| **BrdU** | *5-bromo-2'-deoxyuridine* |
| **CalA** | *calyculin A* |
| **DAPI** | *4'-6-diamine-2-phenylindol* |
| **DDR** | *DNA damage response* |
| **DNA** | *deoxyribonucleic acid* |
| **DSB** | *double strand break* |
| **DSS** | *dextran sulfate* |
| **EdU** | *5-ethynyl-2´-deoxyuridine* |
| **EM** | *electron microscopy* |
| **FACS** | *fluorescence activated cell sorting* |
| **FSC** | *forward-scatter* |
| **HCS** | *High content screening* |
| **HR** | *homologous recombination* |
| **KI** | *knock-in* |
| **KO** | *knock-out* |
| **LOH** | *Loss oh heterozygosys* |
| **MEF** | *Mouse embryonic fibroblast* |
| **MMC** | *mitomycin C* |
| **MMS** | *methyl methanesulfonate* |
| **NSE** | *Non SMC element* |
| **PBS** | *phosphate buffered saline* |
| **PCR** | *polymerase chain reaction* |
| **PFA** | *paraformaldehyde* |
| **ROS** | *Reactive oxygen species* |
| **SCE** | *sister chromatid exchange* |
| **SD** | *SUMO dead* |
| **shRNA** | *short hairpin RNA* |

**siRNA** *short interfering RNA*

**SL** *synthetic lethality*

**SMC** *structural maintenance of chromosomes*

**SSC** *side-scatter*

**ssDNA** *single stranded DNA*

**SV40** *Simian Virus 40*

**UV** *ultraviolet (radiation)*

**WT** *wild type*

**γH2AX** *phosphorylated form of H2A.X at Ser139*

# Introduction

# Introduction

## DNA molecules: a robust yet fallible substrate to store information

Observing the history of life backwards in time, we can certainly conclude that the *DNA strategy* to transmit information through generations has proven to be a rather successful one.

DNA molecules present numerous advantages as an information vehicle: the "information density" of DNA - a measure of the amount of information theoretically recordable on a certain substrate - surpasses that of many digital media currently used today. Its chemo-physical properties, moreover, make it a remarkably stable and robust support (Bancroft, Bowler et al. 2001; Church, Gao et al. 2012).

Despite its intrinsic stability and versatility though, DNA molecules are prone to damage and therefore, alteration of the encoded information.

A plethora of environmental agents, such as ionizing radiations and reactive oxygen species (ROS) as an example, can corrupt the chemical backbone of the molecule, affecting the information stored. In addition, the same outcome may result from the constant *wear and tear* that DNA undergoes during its replication (Groth, Rocha et al. 2007). Despite being the driving force of evolution, mutations and DNA damage often result in negative effects on the survival of organisms. In order to preserve fitness, the issue has obviously to be dealt with, and it is hence of no surprise that a selective pressure for repair strategies has taken place since the earliest stages of life. DNA repair pathways have indeed been described for almost all the different threats that can assault DNA molecules, and cells can implement a variety of cooperative and alternative approaches in order to restore the *status quo ante* after the establishment of damage (Bartek and Lukas 2007; Branzei and Foiani 2005; Sancar, Lindsey-Boltz et al. 2004).

Such is the case for the response elicited by lesions arising at DNA replication - an important source of endogenous damage.

## Replication and cellular DNA metabolism are a major source of replication intermediates.

The complex array of operations necessary for the duplication of DNA molecules, with its intrinsic (and inevitable) imperfection, as well as the action of DNA damaging agents

during the process, may halt replication, and consequently lead to the accumulation of DNA replication intermediates (**figure 1**) (Lönn and Lönn 1985; Lönn, Lönn et al. 1990; Segurado, Gomez et al. 2002; Sogo, Lopes et al. 2002; Mankouri, Huttner et al. 2013).

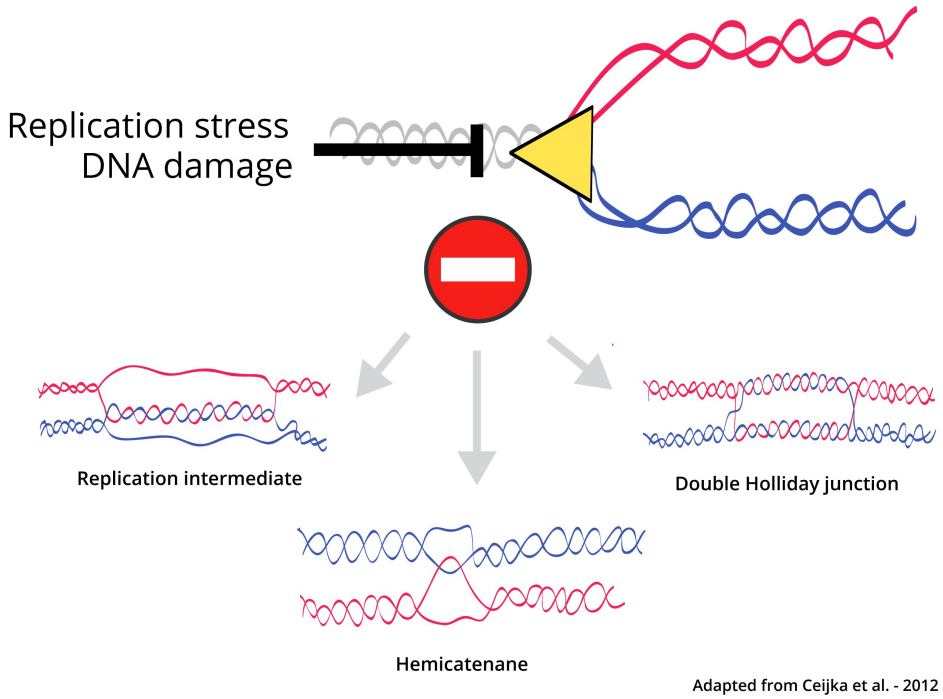



**Figure 1: replication arrest leads to the accumulation of DNA replication intermediates.** Arrested replication forks leave behind DNA replication intermediates, also known as joint DNA species, that may assume different configurations depending on the source of damage that originated them (Aguilera and Gómez-González, 2008).

Some of these structures can lead to joint DNA molecules which can assume different configurations and topologies. These represent a major issue, for they can degenerate, at cell division, in DNA bridges, hampering the correct segregation of chromosomes to daughter cells (Chan, North et al. 2007; Shimizu, Shingaki et al. 2005). In eukaryotic cells, mis-segregation often results in chromosomal aberrations at anaphase: micronuclei and double minutes chromosomes may arise, resulting in gene copy number variations and, potentially, aberrant expression patterns that can lead to malignancy (Chen and Chen 2008; Ogiwara, Kohno et al. 2008; Smida, Baumhoer et al. 2010). Additionally, the breakage of DNA bridges can generate exposed fragments of DNA, prone to recombination, with the risk of producing loss of heterozygosity (LOH) and wreaking havoc on the genome, as in the case of several chromosomal instability syndromes

(**figure 2**) (Gupta, Sahota et al. 1997; Luo, Santoro et al. 2000; Ogiwara, Kohno et al. 2008).

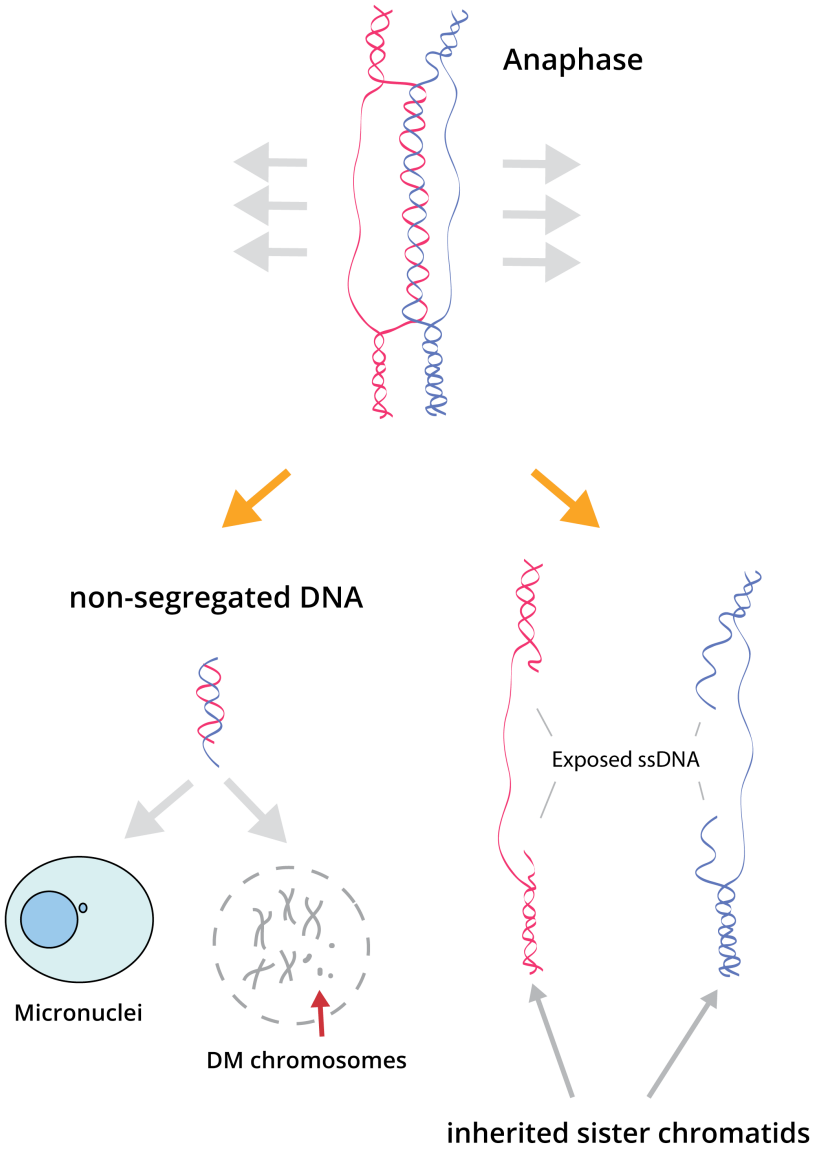



**Figure 2: unprocessed DNA joint molecules may give rise to chromosomal aberrations at anaphase.** When pulled apart at anaphase, intertwined chromatids may undergo breakage. The resulting fragments, which are not inherited by both daughter cells, may give rise to micronuclei or DM chromosomes. Additionally, exposed ssDNA originating from the rupture may promote unbalanced recombination, leading to LOH and gross chromosomal rearrangements.

**Dissolution and resolution deal with accumulated replication intermediates in cells.**

Due to their peculiar topology, replication intermediates are processed in cells by a well-orchestrated enzymatic machinery involving at its operative core topoisomerases, helicases and structure-specific nucleases.

While the first are enzymes catalyzing the under/over-winding of DNA filaments, the second operate by separating two annealed nucleic acid strands using the energy derived from ATP. Members of the third class can instead generate cuts on the DNA molecules by cleaving the phosphodiester bonds between its nucleotide subunits.

Upon the accumulation of replication intermediates, cells can operate the dissolution of these structures (**figure 3**). The final goal of the process, which involves helicases and topoisomerases, is the disentanglement of two sister chromatids of replicated DNA intertwined together, allowing their correct separation at anaphase. The gene *Blm* encodes for the major helicase implicated in dissolution (Cheok, Bachrati et al. 2005), a task that it accomplishes through the functional partnership with topoisomerase IIIα (Yang, Bachrati et al. 2010) and the accessory partners RMI1/RMI2 (Cejka, Plank et al. 2010). Together with WRN, BLM belongs to the family of the ReqQ helicases (Karow, Chakraverty et al. 1997), and both enzymes are the respective etiological causes of the Werner and Bloom syndromes, two autosomal recessive disorders characterized by a striking genomic instability (Mohaghegh, Karow et al. 2001; Shen and Loeb 2000; Yamagata, Kato et al. 1998).

Alternatively, yet to a certain extent cooperatively, replication intermediates can undergo a independent process, known as resolution (**figure 3**), which involves the structure specific nucleases encoded by *Gen1, Slx1, and Mus81* in metazoans (Boddy, Gaillard et al. 2001; Doe, Ahn et al. 2002; Schwartz and Heyer, 2011). The cut produced by these nucleases, similarly to the case of dissolution, results in the separation of the two sister chromatids (Rass, 2013). (**figure 3**) The DSB produced for the separation needs though to undergo repair by homologous recombination (HR). This last process may lead, in half of the cases, to sister chromatid exchanges, bearing the intrinsic potential to generate chromosomal instability and LOH if not correctly performed. A fourth gene, *Slx4*, plays an important role by encoding SLX4, a coordinating *docking protein* orchestrating the

activity of SLX1 and MUS81, at least in human cells (Cybulski and Howlett 2011; Muñoz, Hain et al. 2009; Wyatt, Sarbajna et al. 2013).

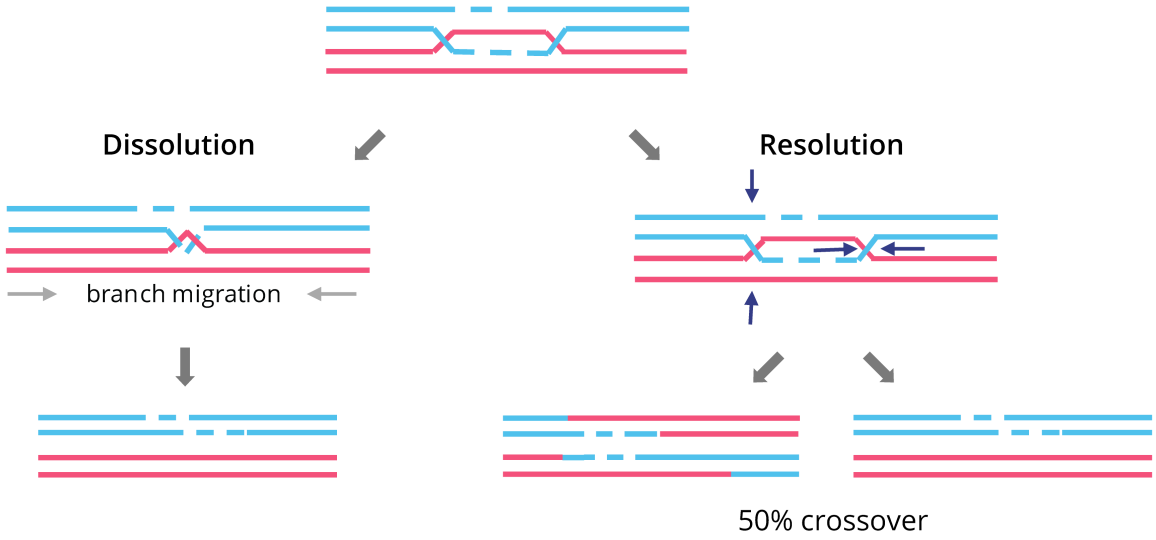



**Figure 3: dissolution and resolution are the housekeeping pathways dealing with accumulated replication intermediates.** Resolution and dissolution are catalyzed by different enzymatic complex which concur to the clearance of toxic DNA replication intermediates

### Dissolution and resolution: a paradigmatic example of redundancy of function.

The monitoring of replication intermediates well summarizes the idea of redundancy, a common feature of many biological processes. Metaphorically, we can imagine cells proceeding along a path, from replication to division, as outlined in **figure 4**.

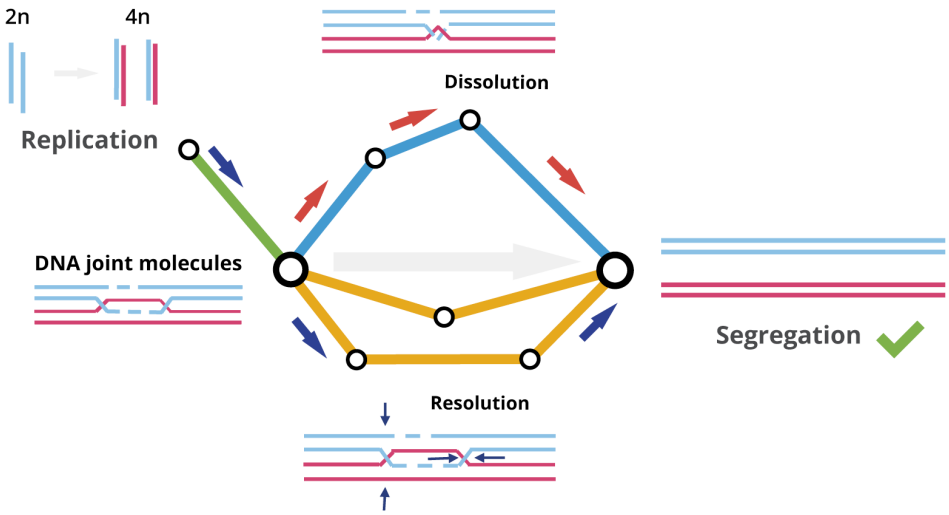



**Figure 4: a trafficking metaphor to illustrate the processing of DNA replication intermediates - #1.** Whenever joint DNA molecules accumulate after replication, they can alternatively undergo resolution or

dissolution, allowing cells to proceed safely towards cell division and avoiding thus potential genomic distress.

At the end of replication, they will have accumulated DNA replication intermediates and in order to continue towards a *risk-free* cell division, two distinct ways are possible. If either of the two is not available, a redundant *backup track* is present to provide an escape route, allowing DNA joint molecules to be trafficked without major issues.

Should both ways be impaired though, as in **figure 5**, the processing of replication intermediates is impeded, and their subsequent accumulation obstacles the physical disentanglement of chromosomes before the onset of anaphase. As a result, during the repartition of DNA to daughter cells, the vast array of chromosomal aberrations previously delineated can arise.

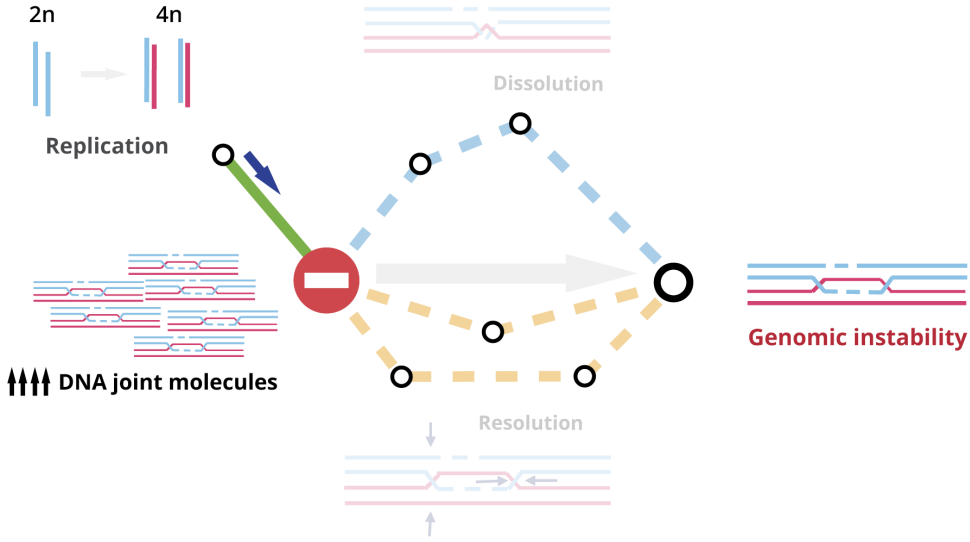



**Figure 5: a trafficking metaphor to illustrate the processing of DNA replication intermediates - #2.** When both resolution and dissolution are impaired, DNA joint molecules accumulate after replication, bringing about the effects commonly observed in several genomic instability syndromes (Imamura, Fujita et al. 2002).

The situation recapitulates what observed in the case of the Bloom syndrome and certain subtypes of Fanconi anemia. The hallmark of *Blm* mutations is a dramatic increase in the frequency of sister chromatid exchanges, resulting from the inoperability of dissolution and the exclusive channelling through resolution of DNA replication intermediates. In agreement with what expected, the impairment of resolution produces genomic instability, too: interestingly, *Slx4* has been recently described as a novel FANC gene (Kim, Lach et al. 2011; Stoepker, Hain et al. 2011). Finally, a wealth of literature

spanning from yeast to Drosophila, highlights the synthetic lethality between *sgs1*/*Blm* mutants and the resolution nucleases, underlining their pivotal functional interplay (Andersen, Kuo et al. 2011; Fabre, Chan et al. 2002).

**A detailed overview of the dissolution machinery**

The protein BLM was discovered and characterized in the '90s as the etiological agent of Bloom's syndrome (OMIM: 210900) (Ellis, Groden, et al. 1995; Karow, Chakraverty et al. 1997). The hallmark feature of Bloom patients is premature ageing, resulting from of a variety of morphological and functional abnormalities affecting several tissues and organs. At the cellular level, mutations in *Blm* typically induce a dramatic chromosomal instability, extensive chromosome breakage and, as mentioned, a manifold increase in the rate of sister chromatid exchanges.

**BLM – TOPIIIα are the effectors of dissolution**

The enzymatic mechanism of dissolution, catalyzed by the complex BLM-Sgs1/TOPIIIα (**figure 6**) (Yang, Bachrati et al. 2010), has been well characterized in yeast. At first, the helicase activity of Sgs1/BLM promotes the branch migration of the crossed junctions between strands (as in **figure 6**), merging them into a single element (Hickson and Mankouri 2011). TopIIIα (Top3a), moving along the DNA strand with Sgs1, takes care of reducing the torsional stress generated by the helicase activity of Sgs1 (Gangloff, McDonald et al. 1994). Finally, by a transient nick on the single DNA strand exposed by the helicase, Top3a allows the disentanglement of the paired filaments of the replication intermediate (Pommier, Pourquier et al. 1998).

**Resolution is promoted by several structure specific nucleases**

Similarly to the case of dissolution, the simpler biology of yeast helped to provide a mechanistic description of the structure-specific nucleases implicated in resolution (**figure 6**). As previously mentioned, several genes are involved in the process in metazoans, the best characterized among them being *Mus81*, for historical reasons. The nuclease Mus81 is a crossover junction nuclease, firstly described in yeast as a gene protecting against MMS and UV radiation-induced damage (Interthal and Heyer 2000). More in detail, the enzyme, which associates to Mms4/EME1 to exert its function (Boddy, Gaillard et al. 2001; Doe, Ahn et al. 2002; Fabre, Chan et al. 2002), has been defined as a

3' flap nuclease, with a specificity towards cruciform DNA structures arising at collapsed replication forks (Gaillard, Noguchi et al. 2003). In comparison to Mus81, our knowledge on the function of Yen1/GEN1 and the complex *Slx1/Slx4* is much more recent and superficial. *YEN1,* the yeast orthologous of human *GEN1,* was originally associated to the processing of Okazaki fragments in replicating cells, suggesting a role as a 5' flap nuclease (Johnson, Kovvali et al. 1998). Only more recently the catalytic activity of human GEN1 towards DNA cruciform structures and Holliday Junctions has been demonstrated *in vitro* (Ip, Rass et al. 2008), the same being true for the complex SLX1/SLX4. These proteins take their name from their *synthetic lethality* (SL) in the absence of Sgs1/BLM in yeast (Mullen, Kaliraman et al. 2001), and they operate as a *holocomplex*. SLX1 participates as the nucleolytic partner, exerting its activity towards 5' DNA flaps and cruciform structures (Fricke and Brill 2003). SLX4 acts instead as a regulatory subunit, coordinating, as *docking platform,* the activity of SLX1 with MUS81 *in vivo* (**figure 6**) (Castor, Nair et al. 2013; Fekairi, Scaglione et al. 2009).

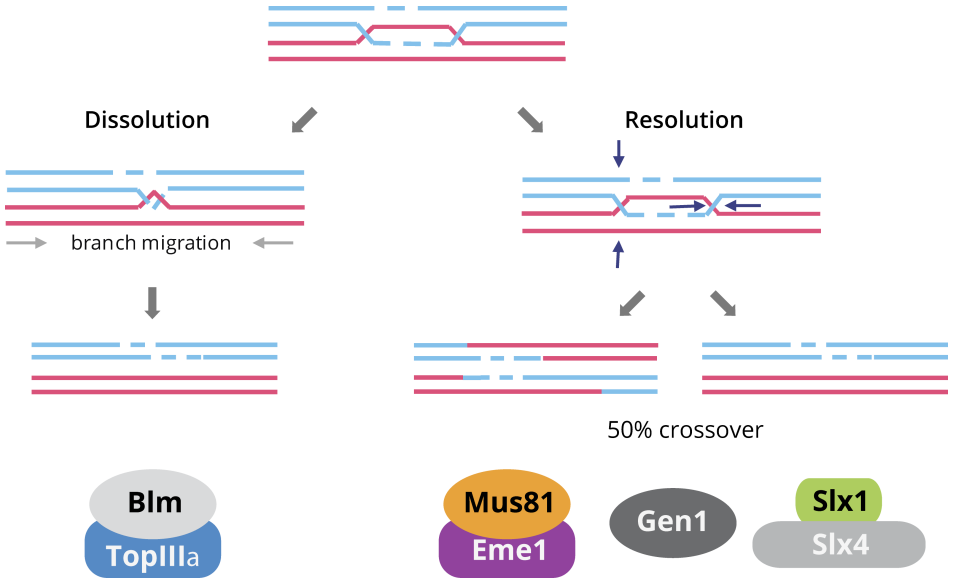



**Figure 6: an overview of the different molecular machineries implicated in the metabolism of Joint DNA species.** Helicases, such as BLM, can catalyze, together with class 1A topoisomerases the dissolution of joint DNA molecules. Resolution involves instead multi-enzymatic complexes, including MUS81 and its functional partner, EME1 (Boddy, Gaillard et al. 2001), GEN1 and the SLX1/SLX4 complex (Svendsen and Harper 2010).

**Dissolution and resolution are poorly characterized *in vivo***

Despite a rather thorough biochemical characterization of their mode of action *in vitro*, these enzymatic complexes are still poorly characterized *in vivo*, especially in higher eukaryotes. Importantly, there's a major gap to fill concerning their interplay and the regulation of their activity, though recently tentative models have been proposed (Matos, Blanco et al. 2011). Due to its strong relevance to cancer, the great interest in dissecting the mechanisms preventing DNA replication intermediates comes as no surprise.

While major attention has been historically dedicated to the direct role of nucleases and helicases, the attention recently drifted towards more unexplored avenues, such as the overall orchestration of dissolution and resolution and its framing inside the cell cycle. The predictable interplay of other proteins in the process represents a vastly unexplored scenario.

Experiments performed in lower eukaryotes pinpointed at a panel of evolutionarily conserved genes, whose mutation phenocopies mutants for the core molecular machinery of dissolution and resolution.

**The SMC5/6 complex – a fundamental genome caretaker**

Among the plethora of enzymes genetically interacting with the dissolution and resolution machinery (**figure 7**), an interesting group of proteins, denominated SMC (an acronym standing for *Structural Maintenance of Chromosome*) stands out for its pivotal role as *chromosome caretakers.*

The members of this family, which comprehends *cohesin*, *condensin* and the SMC5/6 heterodimer*,* are implicated in segregation, transcription, as well as in replication and the repair of damaged chromosomes (Hirano 2006; Losada and Hirano 2005; Nasmyth and Haering 2005; Wendt and Peters 2009).

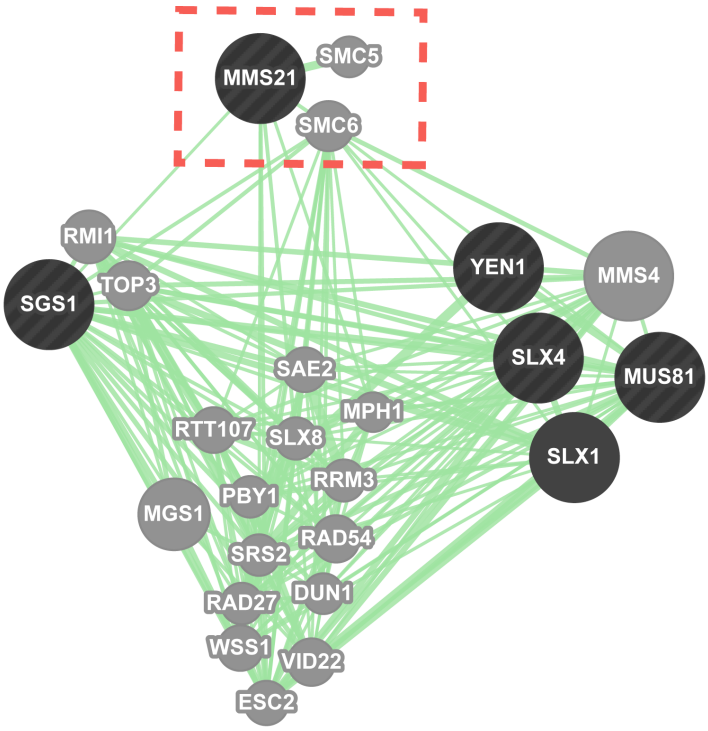



**Figure 7: the SMC5/6 complex genetically interacts with the DNA replication intermediates machinery.** When analyzing the genetic interactions between the resolution and dissolution machineries, the family of genes implicated in the SMC5/SMC6 complex shows up as a genetic *linking hub* connecting the two pathways. (Elaborated from GeneMania.org / © University of Toronto).

A wealth of yeast literature has highlighted, during time, the importance of the SMC5/6 heterodimer in several aspects of chromosomal biology. It is best characterized for its involvement in the correct repair by homologous recombination (Pebernard, McDonald et al. 2004; Torres-Rosell, Sunjevaric et al. 2007). Importantly, SMC5 and SMC6 are essential genes in yeast, while the HR related ones are not (Krogh and Symington 2004). There are additional indications on its functional implication in gene expression regulation (Cuperus and Shore 2002; Dhillon and Kamakaka 2000; Yu, Kuzmiak et al. 2010; Zhao and Blobel 2005), as well as on its fundamental involvement in replication and the dissolution of chromosomes during cell division (Bermudez-Lopez, Ceschia et al. 2010; De Piccoli, Potts 2009; Torres-Rosell et al. 2009).

### The architecture of the SMC5/SMC6 complex

The SMC5/6 heterodimer shares its overall topology and structural features with *cohesin* and *condensin*, the other members of the SMC family. All these contain a central $\alpha$–helical region, at whose ends two globular domains are present (**figure 8**).

Both the N-terminal and the C-terminal globular domain comprehend a walker A and walker B motif, respectively. A third domain, denominated hinge domain, is found in the middle of the helical region. Its name is due to its localization at the point where the SMC proteins fold back on themselves.

The tertiary structure of SMC proteins is such to bring the Walker motifs together, forming an ATPase connected to the hinge via the coiled coil of α–helical stretches.

Similarly to the case of *cohesin* and *condensin* (Arumugam, Gruber et al. 2003; Kimura and Hirano 1997), yeast Smc5/6 possesses an ATPase activity which is specifically stimulated by double-strand DNA (Fousteri and Lehmann 2000).

Noteworthy, point mutations in the ATP-binding domain of SMC6 render cells sensitive to DNA-damaging agents and promote rather than suppress recombination (Ju, Wing et al. 2013; Verkade, Bugg et al. 1999).

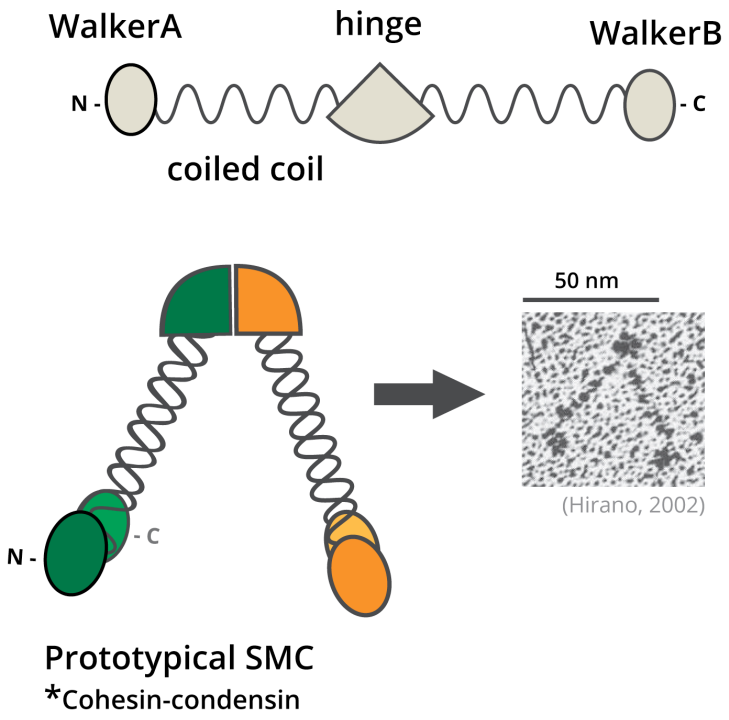



**Figure 8: SMC proteins share common secondary, tertiary and quaternary structures.** *Cohesin*, *condensin* and both SMC5 and SMC6 are formed by α-helical coils linking together two globular domains at the N and C *termini*. All SMC proteins fold back on themselves through a hinge domain in the middle of the α-helical stretch. The prototypical quaternary structure of the SMC heterodimers, as well as an EM image of its conformation, is depicted (adapted from Hirano 2002).

### SMC5/6: a diverse and multitasking complex

The heterodimer SMC5/6 acts as the backbone of a multimeric complex comprising six additional subunits, denominated *non-structural maintenance of chromosome* elements (*Nse*), as in **figure 9** (Fousteri and Lehmann 2000; Fujioka, Kimata et al. 2002; Hazbun, Malmström et al. 2003; McDonald, Pavlova et al. 2003; Morikawa, Morishita et al. 2004; Pebernard, McDonald et al. 2004; Zhao and Blobel 2005).

In budding yeast, Nse proteins are named Nse1, Mms21/Nse2, Nse3, Nse4/Qri2, Nse5, and Kre29/Nse6. In fission yeast instead, Smc6 is identified as Rad18, Smc5 as Spr18, and Nse4 as Rad62 (Fousteri and Lehmann 2000; Morikawa, Morishita et al. 2004; Verkade, Bugg et al. 1999). The complex has a different organization in metazoans. Out of the eight proteins described in lower eukaryotes, only six human orthologous (SMC5, SMC6, NSE1, NSMCE2/MMS21, NSE3/MAGE1 and NSE4) have been shown to form a complex *in vivo* (Taylor, Moghraby et al. 2001; Harvey, Sheedy et al. 2004; Potts and Yu 2005).

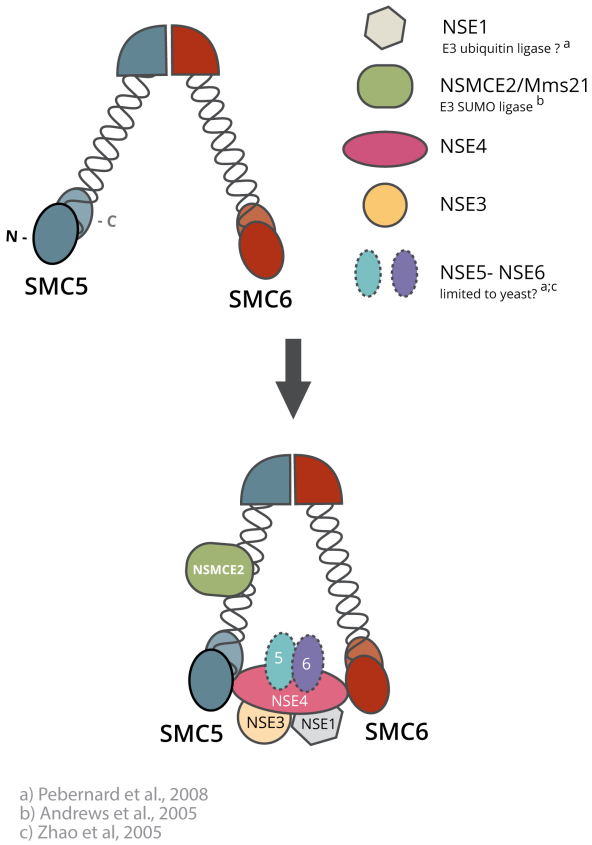



**Figure 9: Several additional subunits associate to the SMC5/6 backbone**. The SMC5/6 complex comprehends a number of associated NSE proteins (6 have been described in lower eukaryotes, with only 4 shared in metazoan). Apart from possessing an ATPase activity (Fousteri

and Lehmann 2000), the associated proteins NSE1 and NSE2/NSMCE2/Mms21 bring to the complex their ubiquitin and a SUMO ligase activity respectively (Pebernard, Perry et al. 2008; Potts and Yu 2005).

**SMC5/6 is an essential complex playing a role in DNA repair**

The biology of the SMC5/6 complex has been mostly characterized in yeast, where both the core elements, as well as some of the associated *Nse* proteins are shown to be essential for cell viability (Fousteri and Lehmann 2000; Fujioka, Kimata et al. 2002; Lehmann, Walicka et al. 1995; McDonald, Pavlova et al. 2003).

Numerous studies demonstrate that cells carrying hypomorphic alleles of these genes show increased sensitivity to a broad spectrum of DNA damaging agents, like ionizing radiation, UV, and *methyl methanesulfonate* (MMS) (Fousteri and Lehmann 2000; Fujioka, Kimata et al. 2002; Hu, Liao et al. 2005; Lehmann, Walicka et al. 1995; McDonald, Pavlova et al. 2003; Onoda, Takeda et al. 2004; Pebernard, McDonald et al. 2004; Torres-Rosell, Machin et al. 2005).

The SMC5/6 complex, moreover, has been associated, in fission yeast, to the correct segregation of chromosomes at mitosis (Torres-Rosell, Machin et al. 2005; Verkade, Bugg et al. 1999). Similarly, budding yeast cells carrying hypomorphic alleles of *smc6* undergo mitotic catastrophe, due to unresolved inter-chromosomal linkages (Farmer, San-Segundo et al. 2011).

A general summary of the phenotypes associated to Smc5/6 mutations is presented in **table 1**.

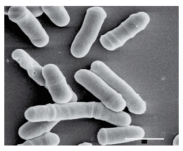
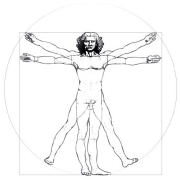
| | Mutation/alteration | Phenotype |
|---|---|---|
|  | Nse1 - RING domain | Growth defects (*S. cerevisiae*) [Santamaria et al. 2007]<br>Increased sensitivity to DNA-damaging agents (*S. pombe*) [Pebernard et al. 2008] |
| | smc5 - 6 (T¨sensitive)<br>smc6 - 9 (T¨sensitive)<br><br>rad18-74 (A151T)<br>rad18.X (R706C) | DNA repair defects [Verkade et al., 1999)<br>Mitotic catastrophes [ibidem]<br>Synthetic lethal with Sgs1 (*S. cerevisiae*) [Torres-Rosell et al., 2005]<br>Synthetic growth defect with Mus81/Eme1 (*S. cerevisiae*) [Torres-Rosell et al., 2005]<br>Defective growth upon UV irradiation and MMS treatmet (Verkade et al., 1999)<br>(Lehmann et al., 1995) |
|  | mms21- CH (E3-dead)<br>mms21-SP<br>nse2 - SA (E3-dead) | Accumulation of cruciform DNA structures upon MMS treatment *(S. cerevisiae)* [Branzei et al., 2006]<br>Telomeric regions mislocalization (*S. cerevisiae)* (Zhao, Blobel, 2005)<br>Increased sensitivity to DNA damaging agent and HU (*S. pombe*) [Andrews et al., 2005] |
| | nse3-1 (hypomorph.) | Increased sensitivity to genotoxic stress (*S. pombe*) [Pebernard et al., 2004] |
|  | hNse2 RNAi | Defects in SC recombination [Potts et al., 2006]<br>Abolished recruitment of cohesin to DSB [ibidem]<br>Abolishment of Smc5 to DSB [Taylor et al., 2008]<br>Increased rates of HR between SCEs upon DNA damage [Potts et al., 2006]<br>Induction of apoptosis [Potts et al., 2005]<br>Delayed mitotic progression [Behlke-Steinert et al., 2009] |
|  | hSMC5-SMC6 RNAi | Delayed mitotic progression (SMC5) [Behlke-Steinert et al., 2009]<br>Increased rates of HR between SCEs upon DNA damage [Potts et al., 2006]<br>Inhibition of telomeres HR in ALT cells [Potts et al., 2007]<br>Loss os SC cohesion in prometaphase [Behlke-Steinert et al., 2009] |

**Table 1**: **mutations affecting the SMC5/6 and associated phenotypes across species.** Mutations in SMC5, SMC6 or associated NSE proteins generally affect DNA repair and the resistance of organisms to DNA damaging agents, as well as the correct segregation of chromosomes at both meiosis and mitosis (Farmer, San-Segundo et al. 2011; Jessberger 2002; Torres-Rosell. 2005; Verkade, Bugg et al. 1999).

## The SMC5/6 complex possesses SUMO ligase activity, provided by NSE2/NSMCE2

Interestingly, among the essential proteins involved in the SMC5/6 complex, Nse1 contains a RING finger domain, analogous to that of E3 ubiquitin ligases, while Nse2/NSMCE2/Mms21 bears a SP-RING domain, related to the SUMO ligases of the PIAS family (McDonald, Pavlova et al. 2003). Whilst the enzymatic activity of yeast Nse1 could only be proven *in vitro* upon the co-presence of purified Nse3 (Pebernard, Perry et al. 2008), different studies, ranging from yeast to human, have confirmed Mms21/NSMCE2 as an active SUMO E3 ligase (Andrews, Palecek et al. 2005; Potts and Yu 2005).

SUMO is a *ubiquitin-like* protein that can be covalently conjugated to targets (Gill 2004; Johnson 2004). Similarly to ubiquitin, the SUMOylation of substrates is catalyzed by a chain reaction involving an E1 SUMO-activating enzyme (AOS1-UBA2), a subsequent E2 conjugating enzyme (UBC9) and finally, an E3 SUMO ligase (such as Mms21/NSMCE2).

The importance of SUMOylation in the context of genomic instability is supported by a wealth of literature, highlighting how numerous proteins participating in DNA repair undergo SUMO modification and SUMO-mediated regulation (Eladad, Ye et al. 2005; Hoege, Pfander et al. 2002; Jackson and Durocher 2013; Pfander, Moldovan et al. 2005; Sacher, Pfander et al. 2006; Saitoh, Pizzi et al. 2002).

**NSE2/NSMCE2 is a SUMO ligase with fundamental implications in DNA repair**

The alternative name for *NSMCE2* in yeast – *MMS21* – recalls its history.

It has been, for a long time, a rather anonymous gene, first described in 1977 as a mutant conferring MMS-sensitivity and a significant increase in spontaneous mitotic recombination in yeast (Prakash and Prakash 1977). Upon the recent discovery of its SUMO ligase activity (Andrews, Palecek et al. 2005) NSMCE2/MMS21 has gained momentum, partially due to the paucity of E3 SUMO ligases encoded in eukaryotic genomes. Additional progress on its characterization has been made, principally in yeast, where the protein was shown to be physically associated to the coiled-coil region of Smc5 (Zhao and Blobel 2005). In terms of its biochemistry, Mms21/Nse2 was shown to autoSUMOylate and promote the SUMOylation of two substrate proteins, Smc5 and Ku70 (Zhao and Blobel 2005). The *S. pombe* Nse2 stimulates instead the SUMOylation of Smc6 and Nse3, but not Smc5 or Nse1 (Andrews, Palecek et al. 2005). If only a handful of substrates for Mms21/NSMCE2 have been characterized during time in both yeast and metazoans, it is now clear that the protein plays a dichotomic role in the SMC5/6 complex. Despite being essential, budding and fission yeast cells bearing SUMO ligase-impairing mutations in NSMCE2 are viable, though they remain hypersensitive to DNA damaging agents (Andrews, Palecek et al. 2005; Zhao and Blobel 2005). These results suggest that the SUMO ligase activity of Mms21 is not required for its essential function, but is important for a fully proficient DNA damage response.

**NSMCE2 is implicated in the prevention of recombination processing of DNA replication intermediates**

Apart from its general implication in DNA repair, yeast *MMS21* has been shown to play a specific and pivotal role in the prevention of recombination and the accumulation of joint DNA molecules arising at replication, or during HR-mediated repair. Yeast *mms21*

mutants, where the C-terminal SP-RING domain is disrupted, accumulate joint DNA molecules at damaged replication forks (Branzei, Sollier et al. 2006).

Importantly, these observations are recapitulated when employing an alternative mutant strain of yeast carrying two *missense mutations* (C200A; H202A) that can disrupt the SUMO ligase activity of the protein (Andrews, Palecek et al. 2005).

In summary, the NSMCE2-mediated SUMOylation regulates the process that counteracts the accumulation of DNA cruciform structures during replication.

**NSMCE2 mutations induce the accumulation of joint DNA molecules (*phenocopying Blm/Sgs1* mutations)**

An important feature of yeast *sgs1/Blm* mutant cells, similarly to metazoans, is the accumulation DNA cruciform structures upon the induction of DNA damage (Fabre, Chan et al. 2002; Kaliraman, Mullen et al. 2001). Differently from higher eukaryotic cells though, these can be directly visualized through 2D electrophoresis gels in yeast (Liberi, Maffioletti et al. 2005). By taking advantage of this technique, different groups could emphasize how yeast mutants for the SUMO ligase activity of Mms21 show the same pattern of joint DNA molecules accumulation as *sgs1* mutants (Bermudez-Lopez, Ceschia et al. 2010; Branzei, Sollier et al. 2006). Considering that Sgs1 is SUMOylated in budding yeast (Lu, Tsai et al. 2010), an intriguing corollary of this *phenotypical overlap* could hence be the functional interplay between the dissolution machinery and the SMC5/6 complex, mediated, possibly, by the SUMO ligase activity of NSMCE2/Mms21.

**The SMC5/6 complex playing a role in dissolution? An open possibility to explore.**

Preliminary experiments failed to show the rather straightforward hypothesis of Mms21 SUMOylating Sgs1 (Branzei, Sollier et al. 2006), leaving a rather undefined scenario around the plausible regulatory role of the SMC5/6 in dissolution.

Several lines of evidence are indeed pointing at this possibility. For instance, mutants for Smc5/Smc6 are synthetic lethal with *sgs1* and *mus81* in yeast, as well as with *mms4***/***Eme1* (the functional partner of Mus81) and *slx4-slx1* (Cost and Cozzarelli 2006; Morikawa, Morishita et al. 2004; Pebernard, McDonald et al. 2004; Torres-Rosell, Machin et al. 2005). Moreover, as previously discussed, SMC5/6 mutations affect the correct segregation of chromosomes at mitosis and meiosis, similarly to *sgs1/Blm* mutants

(Bermudez-Lopez, Ceschia et al. 2010; Branzei, Sollier et al. 2006). Additionally, mutations in *mms21*, a preeminent *functional element* of the SMC5/6 heterodimer, induce an increase in recombination rates (Prakash and Prakash 1977), again phenocopying *sgs1* mutants. Finally, additional evidence from *S. pombe* links the functional integrity of the SMC5/6 complex to the prevention of DNA replication intermediates (Lindroos, Strom et al. 2006).

We therefore cannot but conclude that further investigation on the *genetic equivalence* between dissolution mutants - SMC5/6 would indeed be a sensible and enthralling endeavour which could help shedding a light on its physiological role. All this being particularly true in higher eukaryotes, where a thorough characterization on the specific proteins implicated is still vastly lacking.

For this reason, we envisaged the possibility of developing a novel set of murine models, aimed at demonstrating the hypothesis of a role for the SMC5/6 multimeric complex in regulating the accumulation of joint DNA molecules.

# Objectives

## Objectives

- To analyze the role of the SMC5/6 complex in mammals by the use of a SUMOylation deficient allele of NSMCE2.
- To explore the genetic interaction between the SMC5/6 complex and structure-specific nucleases involved on the resolution of intermolecular DNA links.
- To investigate a potential role of mitotic kinases and structure-specific nucleases on the phenomenon of “chromosome pulverization” (CP).
- To generate cellular systems where the activity of structure-specific nucleases can be stimulated at will.

# Materials and methods

# Materials and methods

## Mice handling

### Genotypings and maintenance

All animals used during the development of this thesis were kept in the animal facility of the Spanish National Cancer Research Center, according with the Spanish animal protection law (R.D. 1201-2005) and the European directive 86/609/CEE established to regulate the standards of animal care

In order to genotype the animals, DNA was extracted from tail samples digested during 16hrs at 56°C in a lysis buffer composed of 100mM NaCl, Tris-HCl pH 8,00 20mM, EDTA 10mM, SDS 0,5%, proteinase K (Roche) 400 µg/ml. Proteins from the lysates were salted out with saturated NaCl and DNA was precipitated with isopropanol 100%, washed with ethanol 70% and resuspended in 10 mM Tris-Cl, pH 8,5. Genotyping PCRs were performed in a reaction mix composed of 200 µM dNTPs, 1 mM $MgCl_2$, 6mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8,8, 1U EcoTaq polymerase (Ecogen/Bioline), 1µM of each primer, ~ 100 ng of genomic DNA.

### PCR oligonucleotides

***Nsmce2$^{SD\text{-}neo}$***: primers amplify a band of 360bp for the wt allele and a band of 560 bp for the NSMCE2$^{SUMOdead\text{-}neo}$ allele

FWD: GGTGGGACTGGAGTATTGGA

REV: TATGTGCGTTGAGTGTGCAA

REV-neo: CAGAAAGCGAAGGAGCAAAG

***Mus81$^{KO}$*:** primers amplify a band of 1087 bp for the *KO* allele, and a band of 764bp for the *wt* allele.

Mus81 wt FWD: ACTCAGCACCACACCGTCCTC 3’

Mus81 wt: REV: CCCACCCAGCCTCAAGCAG

Mus81 KO FWD: CATTGCGCTCCCTCCAACGGTA

Mus81 KO REV: GGCCAGCTCATTCCTCCCACTCATG

**$Smc6^{S994A\text{-}neo}$:** primers amplicons obtained with the following primers

FWD: CCCTGGTAAAGCATCCTAACT

REV: CAGGCAAACGCTCAAGATGTAC

were subjected to restriction digestion with NaeI (NEB) providing a digested product in the presence of the point mis-sense mutation.

***$Nsmce2^{lox}$*:**primers amplicons resulted in a band of 150 bp in the case of the *wt* allele, while the *floxed* allele results in a band of 300 bp

FWD: ATTAGAAAATAATGATCCTAGGAAACATTAATTCTGCAACTCGTTGTT

REV: TAAATACTCATGCCTTTCTTACCCCGTCTTTCT

***$Slx4^{lox}$*:** primers amplify a product of 550 bp in the case of the *floxed* allele and a band of 480bp in for the *wt*:

FWD: CTTGTGGACTTGGCAGTGG

REV: GGCTGCTAGGTACCAGGTCC

***$Gen1^{KO}$*:** the wt PCR product results in a band of 415 bp; the KO allele gives rise to a band of 384 bp.

FWD: TTTCAGTGTTGCTTTCTGCAA

$REV^{wt}$: GGCCACTGGCATTAAGGTAA

$REV^{KO}$: GGGTTATTGAATATGATCGGAAT

**Survival curves**

At least 15 adult animals (>14 weeks) for every genotype of interest were maintained for each survival analysis. The age of death was recorded for each animal and reported on a Kaplan-Meyer curve with GraphPad™ (Prism Software).

**Immunohistochemistry**

Tissue samples were embedded in formalin and stained with hematoxylin/eosin, following the protocol routinely used at the CNIO pathology unit.

### Micronucleation/polynucleation scoring - liver sections

Liver sections embedded in formalin were subjected to hematoxylin/eosin staining and analyzed for the presence of micronuclei/polynucleated cells. At least 3 animals were scored for each experimental genotype. The number of aberrancies scored were plotted as a percentage over the whole sample with GraphPad™ (Prism software).

### Azoxymethane-dextran sulphate induced tumorigenesis

6/8-week-old experimental mice and wild-type counterparts were injected intraperitoneally (i.p.) with 12,5 mg/kg AOM. After five days 2,5% DSS was given in the drinking water over five days, followed by 16 days of regular water. This cycle was repeated twice and mice were sacrificed ten days after the last cycle. Colons were removed, flushed with PBS, fixed as "Swiss-rolls" in 4% paraformaldehyde at 4°C overnight and screened for adenomas. Results were plotted with GraphPad™ (Prism software).

## Cellular biology

### Cell culture

Unless otherwise specified, all cell lines were cultured in DMEM (4,5g/l glucose; L-Gln) (Lonza, CH) complemented with 10% or 20% inactivated fetal bovine serum (FBS) (Lonza) and a mixture of penicillin and streptomycin (Gibco/Invitrogen/Life technologies, Carlsbad-CA). Cells were grown in 5% $CO_2$ - 37°C, except for murine fibroblasts, grown in hypoxic conditions (5% $O_2$).

### Production of MEFs (mouse embryonic fibroblasts)

Female and male mice of the desired genotypes were mated until vaginal plugs were visible. At 13,5 days of gestation (unless otherwise specified) females were sacrificed and embryos were extracted and deprived of embryonic liver and spleen. The remainders were thus chopped with a sterile blade and incubated for 10 minutes in 1 ml trypsin 0,25% (Gibco). The resulting mixture was disaggregated by mechanical pipetting and trypsin was neutralized with 9ml of DMEM 15% FBS. Cell suspensions were then incubated in hypoxic conditions and media changed the following day, to eliminate dead cells and undesired, non attached contaminant cell subtypes.

**Isolation of splenic B lymphocytes**

Splenectomy was performed in mice aged 6/8 weeks. Whole spleens were squeezed in PBS and supernatants treated with a hypotonic solution (ACK lysing buffer, Lonza) during 10 minutes. Supernatant was filtered through a 40 µm strainer and the remaining solution centrifuged at 350g during 5 minutes. The precipitate was resuspended in 900 µl of PBS to which 80 µl of αCD143-conjugated magnetic beads were subsequently added (Miltenyi Biotech, Germany). The mixture was incubated for 30 minutes at 4°C and after this time, cells were washed, resuspended in 1 ml PBS and transferred to a magnetic separation column (MS columns, Miltenyi) attached to a magnetic scaffold (OctoMACS separator, Miltenyi). The non-retained fraction, containing a pool enriched for B-cells, was retained and maintained in culture in RPMI medium (Euroclone/Lonza) supplemented with 10% FBS, 1% penicillin/streptomycin, 2mM glutamine (Gibco/ Invitrogen), MEM-Non-Essential amino acid solution (Lonza), 1mM sodium pyruvate (Gibco/Invitrogen), 50 mM β-mercaptoethanol (Gibco/Invitrogen), HEPES 10 mM (Lonza) and 25 mg/ml LPS (Sigma Aldrich, St. Louis, MO) in order to stimulate cell proliferation.

**Cell proliferation curves**

12500 cells were seeded in triplicate in 24 well plates. Six hours after seeding, they were washed in PBS and fixed at timepoints in 2% glutaraldehyde/PBS for 15 minutes. After washing with water, fixed cells were stained with a 0,1% crystal violet water solution for 20 minutes on a rocking platform. After washing three times with water, plates were air dried, and crystal violet eluted with 10% acetic acid for 20 minutes on a rocking platform. The absorbance at 595 nm for each sample was assayed on a spectrophotometer (BioRad) and log values over starting points were plotted.

**Micronucleation/polynucleation quantifications**

Cells were seeded at a density of 5000/ cell/well in 96 well plates in triplicate and grown for 72 hrs in the presence of increasing concentrations of MMS (ranging from 0,025mM to 0,5 mM). They were then washed in PBS, fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature, rinsed with PBS and stained with a solution of DAPI and HCS Deep Red CellMask™ dye (Invitrogen) to stain the cellular body and chromatin differentially. Cells (n>100) were scored for the presence of ≥ 1 micronucleus or nucleus and results were plotted as normalized over the minimal count in the control condition.

**Immunofluorescence/*high-throughput* immunofluorescence**

Either 10000 cells (96 well plates) or 30000 cells (24 well plates) were seeded and let settle for 16 hrs. Attached cells were then treated according to the appropriate experimental protocol. They washed 1X with PBS and fixed in 2% paraformaldehyde/PBS for 10 minutes at room temperature. Fixed cells were then washed in PBS twice and permeabilized with a buffer containing 0,1% TritonX-100 in PBS (Sigma-Aldrich) for 5 minutes at room temperature.

Where needed, washed cells were fixed and pre-extracted with a mSTF/CSK buffer to reduce the aspecific cytoplasmic signal. The STF buffer is composed as follows: 150mM 2-bromo-2-nitro-1,3-propanediol, 108mM diazolidinyl urea, 10mM sodium citrate, 50mM EDTA pH 5,7. The CSK is instead composed by 10mM PIPES pH 6,8, 100mM NaCl, 300mM sucrose, 3 mM $MgCl_2$, 1mM EGTA, 0,5% Triton X-100.

Fixed an pre-extracted cells were then permeabilized with a permeabilization buffer (100mM Tris-HCl pH 7,5, 50mM EDTA, 0,5% Triton X-100) and saturated in a blocking solution (2,5% BSA, 0,1% Tween-20, 10% goat serum) for 30 minutes at room temperature.

Cells were then incubated in primary antibody at 4°C for 16hrs, washed in PBS three times for 10 minutes at room temperature and decorated with Alexa Fluor® secondary antibodies (Invitrogen). After three additional washes in PBS, samples were stained with DAPI and mounted.

High throughput immunofluorescence was performed with the standard protocol described above, but using 96 well plates with flattened transparent bottom (Greiner Bio-one) instead of regular plates. Images from stained cells were then acquired automatically with the Opera™ High Content Screening platform (Perkin Elmer, Waltham, MA) in use at the Imaging facility of the CNIO. Quantifications and data analysis were performed with the Acapella® Imaging and analysis software.

**HCS EdU proliferation assays**

When EdU (5-ethynyl-2-deoxyuridine) staining was required to assay cell proliferation in high throughput, cells were treated with EdU ranging from 30 minutes to 2 hours, depending on the cell type and its replication rate. After EdU incorporation, cells were

fixed with 4% paraformaldehyde/PBS at room temperature for 10 minutes and processed according to the protocol provided with the Click-it EdU cell proliferation assay (Life Technologies).

**BrdU incorporation/metaphase spreads**

In order to discriminate actively proliferating cells, BrdU (5-bromo-2'-deoxyuridine) was added to culture medium at a concentration of 10μM. Karyomax Colcemid (Life Technologies) was then added to cells for 2 to 5 hours (depending on the cell type) at a final concentration of 100 ng/ml and metaphase-arrested cells were harvested and resuspended in 75mM KCl for 20 mins. Few drops of a freshly prepared fixing solution (3:1 methanol/acetic acid) was then added to the resuspension. Cells were then centrifuged and subsequently resuspended in the fixing solution, repeating this latter process three times. The cellular pellet obtained was spread on microscopy slides and BrdU detection was performed by denaturing the samples with 1,5M HCl for 30 minutes at room temperature. Slides were then washed three times in PBS and blocked in a solution of 1% BSA/PBS-Tween 0,1% for 30 minutes at room temperature. Finally, slides were decorated with a monoclonal αBrdU antibody (Millipore) for 1 hr at RT and a secondary AlexaFluor® anti mouse antibody.

**Sister chromatid exchange assays**

When scoring for sister chromatid exchanges, actively proliferating MEFs or B lymphocytes were exposed to BrdU at a concentration of 10μM for 5 hours (B cells) or 12 hours (MEFs) and aphidicholin 0,5 μM for 16 hours.

Metaphase spreads were produced according to the protocol outlined above and subsequently incubated with Hoechst 33258 at a concentration of 10 μg/ml in PBS for 30 minutes at 37°C. After rinsing in SSC 2X, cells were exposed to UV (λ = 352 nm) for 60 min) and rinsed in SSC 2X. Finally, they were stained with 3% PBS in Gurr's buffered solution for 5 minutes at RT, washed and visualized at the microscope.

**Colony forming assays**

MEFs were seeded in 6-well plates at a density of 12500 cells/well in triplicate. Where replication challenging was pursued, they were exposed to a high dose of either MMS (final concentration of 10 mM) or mytomicin C (MMC) (0,5 μg/ml to 1 μg/ml for 1hr).

Cells were then washed in PBS and grown for up to 15 days. The colonies obtained were thus fixed in a 1,5% methylene blue/ethanol solution and counted after extensive washing.

### Cell transfection - lentiviral and retroviral transductions

Transfections for transient overexpression and viral productions were performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's standard protocols.

Viral particles for transduction were produced in 293T cells, transfected with either a retroviral packaging vector (pCl-Eco) or a third generation lentiviral platform based on the pMDLg/pRRE-pRSV.Rev-pMDG VSV-G packaging vectors (Dull, Zufferey et al. 1998).

At 48 hours post transfection, the supernatant from the packaging lines was collected, filtered and added on top of experimental cells seeded for the purpose, together with 6µg/ml polybrene.

## Molecular biology/biochemistry

### RNA extraction and gene silencing analysis by RT-PCR

Roughly 1 million cells were usually harvested by trypsinization and pelleted by centrifugation. The pellet obtained was resuspended in Trizol (Invitrogen) and processed according to the producer’s standard protocol. Gene silencing was The RNA obtained was processed using the SuperScript III Platinum kit (Invitrogen) and the cDNA obtained quantified by real time PCR using the following oligonucleotides:

GAPDH FWD: CATGATGGCCATGAGGTCCACCAC

GAPDH REV: GCCACCCAGAAGACTGTGGATGGC

Murine *Slx4* FWD: ATGGAGTGCAAGACTAAGGGG

Murine *Slx4* REV: TGTGGCAAGGGAATTTCTCCT

Murine *Gen1* FWD: CCCGAGTCAGAAATGGAGTCCA

Murine *Gen1* REV: CGCTTCTTCCATTGTAAGGAGGC

Measurements were normalized to the GAPDH levels of each samples and plotted as fold changes over controls.

**Protein extraction/SDS page electrophoresis/Western blot/Immunoprecipitations**

Protein were extracted from harvested cells lysed in a protein lysis buffer (50 mM Tris-HCl pH 7,4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 270 mM sucrose; 1% Triton-X100) supplemented with a mix of protease inhibitors (Sigma-Aldrich) and Benzonase® (Merck).Lysates were thus centrifuged at 16000g for 40 minutes at 4°C to pellet cell debris.

Supernantants were thus quantified/normalized using the Bradford method and directly loaded on gels or processed for immunoprecipitations (IP). These where routely performed by incubating quantified/normalized protein lysates with primary antibodies against the epitope of interest, together with proteinA/G conjugated Dynabeads® (Life Technology) for 16 hrs at 4°C. IP samples were loaded on 4%-12% SDS-PAGE gradient gels after heat-mediated denaturalization in NuPage loading buffer (Life Technologies). Protein gels were then transferred on ECL Hybond™ nitrocellulose membranes and incubated in blocking buffer (10% skimmed milk in TBS/Tween-20 0,05%) for 45 minutes at room temperature. Primary antibody incubation for Western blot detection was routinely performed at 4°C overnight. Decorated membranes were incubated in HRP-conjugated secondary antibodies for 1hr at RT and protein levels revealed using the SuperSignal West Pico protein detection kit (Pierce).

**SUMOylation assays**

Stable 293T FLP-in cells overexpressing NSMCE2$^{WT}$ or NSMCE2$^{C185S;H187A}$ were transfected with constructs encoding for SUMO1-GFP or FLAG-SUMO2 using Lipofectamine 2000 (Invitrogen). 48 hours post-transfection, cells were harvested and processed as following: for SUMO1-GFP overexpressors, lysates were treated accordingly to the instruction of the μMACS GFP Isolation Kit (Milenyi Biotec). In the case of FLAG-SUMO2 overexpression, cells were lysed in the protein lysate buffer previously described supplemented with 20mM N-ethylmaleimide. Precleared lysates were thus normalized and immunoprecipitated with magnetic anti-FLAG beads (Sigma-Aldrich) for 4 hrs at 4°C.

After three washes performed with the lysis buffer, the immunoprecipitated samples were denatured in NuPage buffer and loaded on gradient 4-12% SDS-PAGE gels.

**STREPT-purification of SUMOylated proteins**

OneStrept©-tag mediated purification of SUMOylated proteins was attained using the MagStrep type2 HC beads (IBA lifesciences - Germany) using overexpressing cells processed as outlined in the producer's standard protocol.

**kDNA decatenation assays**

Nuclear extracts of murine fibroblasts were prepared from 100 mm confluent petri dishes. Cells were scraped into medium and pelleted at 800g for 3 minutes at 4°C. The cellular pellet obtained was then resuspended in 5 ml of ice cold TEMP buffer (10 mM Tris-HCl, pH 7,5, 1 mM EDTA, 4 mM MgCl2, 0,5 mM PMSF) and the clump dispersed by pipetting. After an incubation of 10 minutes on ice and additional centrifugation step, the pellet obtained was resuspended in 20µl of TEP buffer (same as TEMP but lacking MgCl2 ). 20µl of NaCl 1M were thus added to the resuspension and the mix was incubated for 1 hr on ice. After an additional centrifugation step of 15 minutes at 16000 g, 5µl of supernatant were employed for the decatenation assay.

The reaction mix consisted of 0.1 ug KDNA (final volume of 20ul), 50 mM Tris–HCl (pH 8), 120 mM KCl, 10 mM MgCl2, 0,5 mM each of DTT and ATP and 30 ug BSA/ml (topo II reaction buffer TG4040).

The reactions were incubated for 15 min at 37o C and terminated with 0,1 volumes of stop buffer (5% sarkosyl, 0.025% bromophenol blue, 50% glycerol). Half of the mixture was then loaded on a 1% agarose gel and the result visualized under UV light.

**Antibodies**

Antibodies used in this work included:monoclonal α-actin (Sigma Aldrich A5441); polyclonal α-53BP1 (Novus 100-304A2); polyclonal α-MUS81 (a kind gift of R. Hakem, Ontario Cancer Institute); polyclonal α-NSMCE2/MMS21 (self raised); monoclonal α-BrdU (Millipore); monoclonal α-STREPT-tag (Novagen); α-FLAG M2 (Sigma-Aldrich); polyclonal α-SMC5 (Santa Cruz Biotechnologies, Santa Cruz, CA); polyclonal α-hSMC6 (kindly provided by A. Lehmann, University of Sussex); monoclonal α-ER (CNIO monoclonal antibody unit); monoclonal α-TopII (Topogen).

# Results – part I

# Results - part I

## A role for NSMCE2-dependent SUMOylation in the activity of the SMC5/6 in metazoans

### Generation of mouse models for *Nsmce2* - preliminary validation

When starting this project, we met with a first issue to overcome: the lack of mouse models for *Nsmce2*.

As a first tentative approach, a gene trapped *Nsmce2*$^{GT/GT}$ mouse strain was generated in the lab (Jacome et al. unpublished), which resulted being early embryonic lethal in homozygosis, recapitulating the lethality observed in *mms21*$^{\Delta}$ yeast cells (Giaever, Chu et al. 2002).

Alternatively, we decided to follow the lead of yeast literature and looked for solutions to impair the function of NSMCE2 avoiding the early lethality. A mutant strain was thus generated, bearing two *mis-sense* point mutations reported, in the *S. pombe*, to impair the SUMO E3 ligase activity of Nse2 (Andrews, Palecek et al. 2005). The *transgenesis* strategy employed is depicted in **figure 10**.

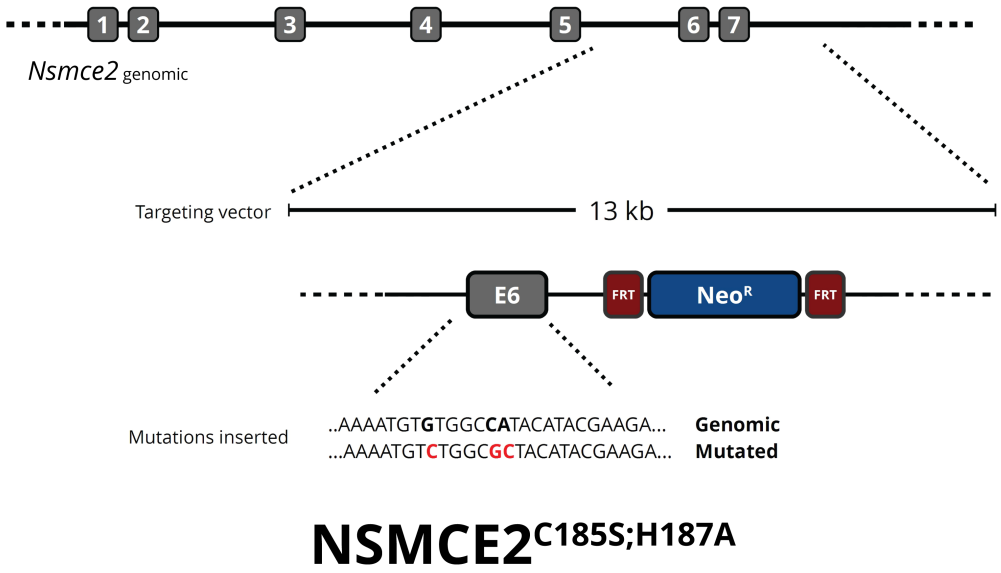



**Figure 10: Targeting strategy for the generation of the *Nsmce2*$^{SD}$ strain**. The C185S; H187A mutation was inserted into exon 6 by a recombination vector bearing Ex.6 and carrying an FRT-neo$^{R}$ cassette. The mouse strain we employed carried the neo$^{+}$ allele.

As outlined in *Duan, Sarangi et al. 2009*, two key Cys and His residues, located at the conserved catalytic core of the SP-RING domain of NSMCE2 and presenting a striking evolutionary conservation, were mutated to Ser and Ala respectively (**figure 11**).

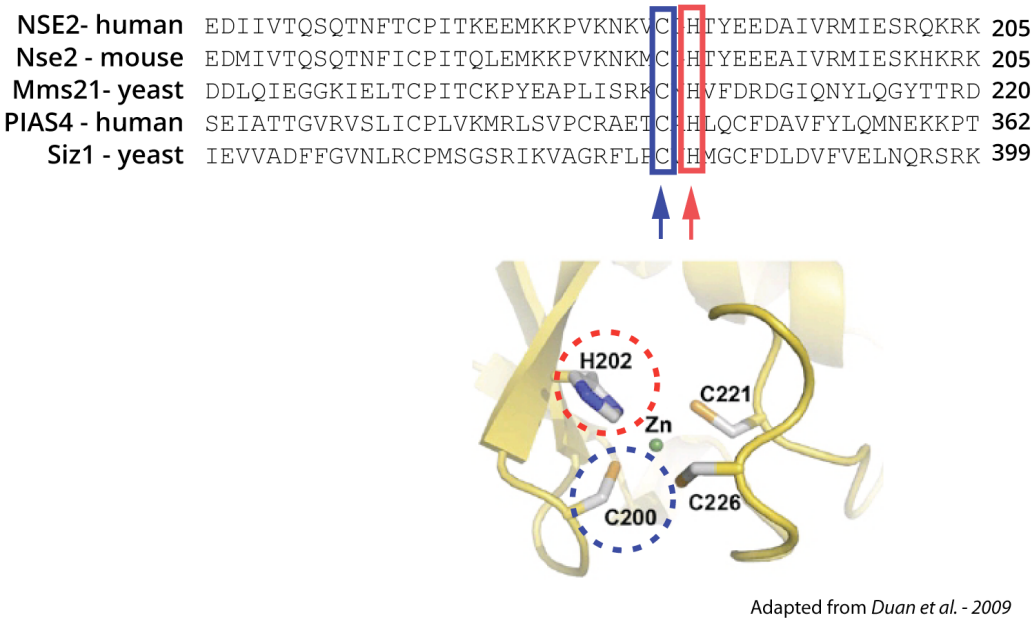



**Figure 11: the SP-RING catalytic core is highly conserved throughout evolution**. The two Cys and His residues mutated in the *SUMO-dead* strain reside in the active site of the SP-RING domain of NSMCE2. Their functional relevance is corroborated by their striking conservation throughout evolution and among other SUMO ligases such as PIAS4 and PIAS1-Siz1. The two residues provide the coordination site for the binding of $Zn^{2+}$, a fundamental cofactor in the catalysis of SUMO conjugation.

In order to prove the validity of our strategy, we cloned the *Nsmce2*$^{WT}$ and the SUMO-dead (SD) version of *Nsmce2* (*Nsmce2*$^{SD}$) from RNA extracts obtained from our mouse model.

We then overexpressed both the NSMCE2$^{SD}$ and the NSMCE2$^{WT}$ proteins in 293T cells, together with tagged versions of either SUMO1 or SUMO2, performed immunoprecipitations against the tags employed and verified the reported auto-SUMOylation of NSMCE2 as a functional readout of the SUMO ligase activity of the protein (Andrews, Palecek et al. 2005; Zhao and Blobel, 2005).

As in **figure 12**, NSMCE2 is able to conjugate both SUMO1 and SUMO2 upon overexpression.

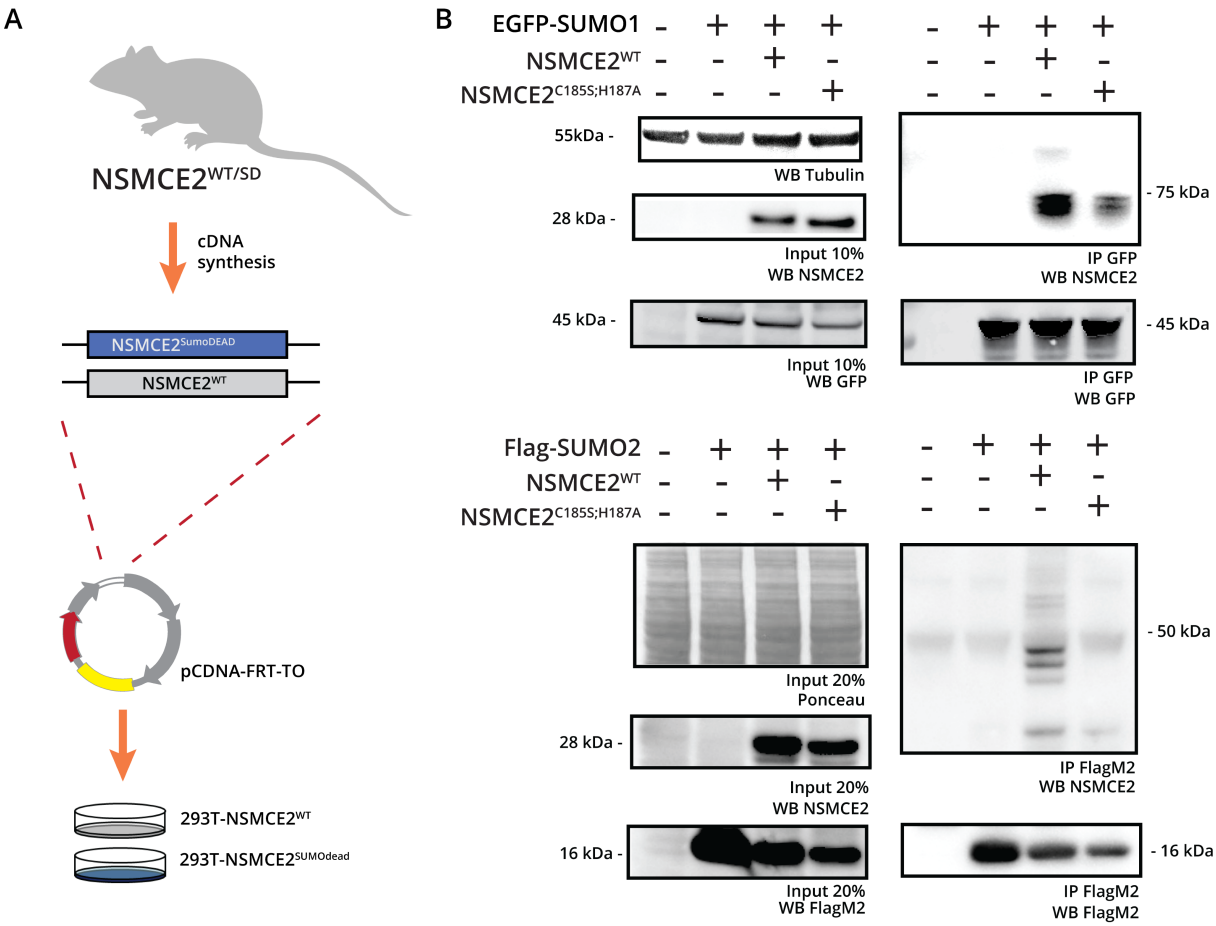



**Figure 12: the C185S; H187A substitutions impair the SUMO2 ligase activity of NSMCE2 *in vitro*.** The experimental strategy followed is outlined. (A) We cloned the cDNA of NSMCE2 (either in its wild-type or *SUMO-dead* versions) in a mammalian expression vector. Stable expressing cell lines for both variants were thus generated. (B) We overexpressed in NSMCE2-cells either SUMO1 or SUMO2, with a GFP or a FLAG tag, respectively. After performing IPs against either of the tags, we detect the *auto-SUMOylated* form of NSMCE2 by WB.

Whilst the *SUMO-dead* mutations negatively affected the protein activity towards SUMO1, it virtually abrogated its SUMO2 ligase catalysis. (**figure 12 - B**).

It is worth underlining how these experiments were performed by overexpressing both the mutated and the wt murine proteins in a context where human NSMCE2$^{WT}$ was expressed at physiological levels. It remains thus possible that the partial activity observed with SUMO1 could be attributed to the activity of the endogenous protein.

**Strategies to define the NSMCE2-associated SUMOylome.**

A first goal of our work was to define the NSMCE2-associated SUMOylome. Despite the presence of only few E3 conjugating enzymes in metazoans, at today's date a global characterization of the proteome fraction specifically modified by NSMCE2 is indeed still missing, and only few targets of the proteins have been described (Pebernard, Wohlschlegel et al. 2006; Potts and Yu, 2005; Zhao and Blobel, 2005). Our attempt aimed at broadening our perspective on the physiological role of NSMCE2, so to narrow our attention on a list of protein candidates for further functional characterization. As outlined in **figure 13**, we generated several different tagged versions of SUMO1 and 2. All clones were inserted in a third-generation lentiviral vector that allowed for antibiotic selection (*blasticidin*) as well as for GFP-expression enrichment via cell sorting.

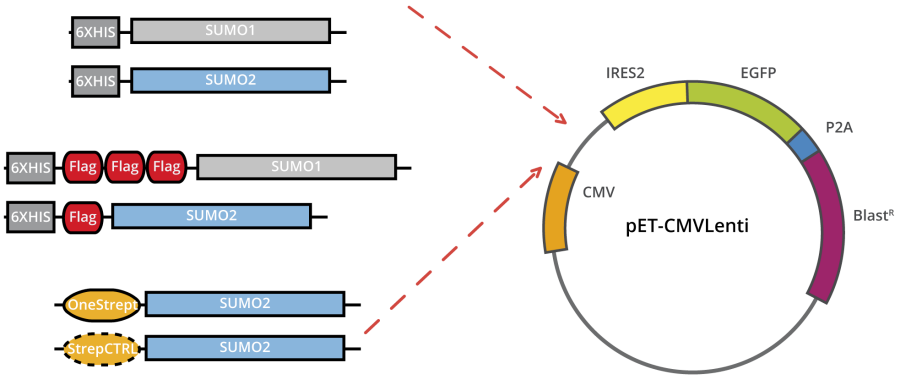



**Figure 13: SUMO overexpression strategies designed to define the NSMCE2 associated SUMOylome.** The murine sequences of SUMO1 and SUMO2 were cloned downstream a 6XHIS tag, a 6XHIs-FLAG/ 6XHis-3XFLAG or a streptavidin tag into a third-generation lentiviral vector allowing for antibiotic selection and fluorescence-mediated sorting.

We transduced SV40$^{T121}$- immortalized MEFs obtained from *Nsmce2$^{SD}$* and *wt* embryos, as in the pipeline diagram of **figure 14**.

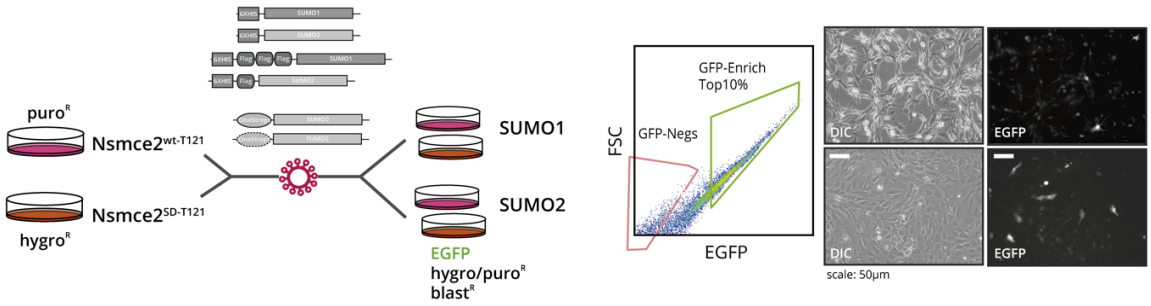



**Figure 14: Experimental pipeline for SUMOs overexpression in murine fibroblasts.** MEFs from the *SUMO-dead* and WT control strains were immortalized with SV40$^{T121}$. After immortalization and drug-

mediated selection of infected cells, these were transduced with one of the different tagged versions of SUMOs. *Blast* and *puro*/*hygro*-resistant cells were then sorted to enrich for the top 10% *expressors*.

The different tagging strategies notwithstanding (and despite the varied purification methodologies employed), we typically encountered the problem of boosting the SUMO levels in murine fibroblasts, a cell type that is often refractory to exogenous protein overexpression (Palmer, Rosman et al. 1991).

Additionally, we noticed a negative effect on cell viability, presumably due to free SUMOs over-expression. This detrimental effect has indeed been reported in different model systems (Rytinki, Finland et al. 2013) underlining how a tight control of SUMO levels is fundamental for cellular homeostasis (Bawa-Khalfe and Yeh 2010). **Figure 15** illustrates the typical outcome of a purification experiment, aimed at enriching for the STREPT-conjugated fraction of the proteome.

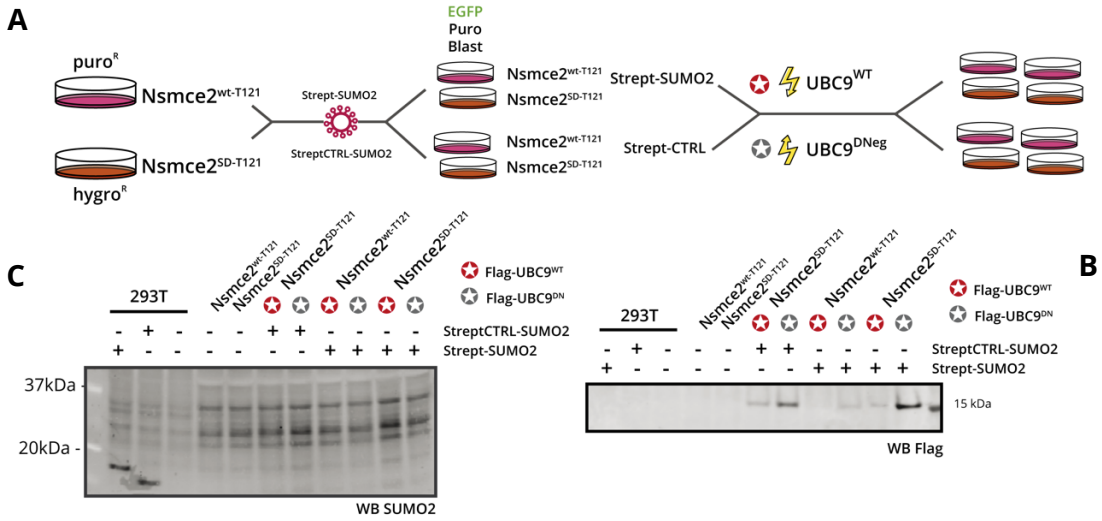



**Figure 15: Experimental pipeline to purify and enrich the NSMCE2 associated SUMOylome.** (A) Immortalized *Nsmce2*$^{SD}$ or *wt* MEFs were transduced with a STREPT-tagged version of SUMO2 or its relevant control. After drug selection and GFP-mediated expression enrichment, they were transiently *electrofected* with either a WT or a DN form of FLAG-UBC9, the SUMO E2 conjugating enzyme (panel B). (C) Despite the GFP-mediated enrichment, free SUMO2 was virtually undetectable in MEFs total lysates.

In conclusion, despite having confirmed the effective *ligase activity* impairment for NSMCE2$^{SD}$ *in vitro*, we were unable to provide a systematic description of NSMCE2 targets in mammalian cells with the strategy undertaken. Additional possibilities are indeed conceivable to address the issue. As an example, recent developments have resulted in optimized antibody-mediated purification strategies to detect endogenous

SUMO targets (Becker, Barysch et al. 2013). Alternatively, novel molecular modifications of endogenous SUMO proteins have allowed to provide a high resolution characterization of global SUMOylation in cells (Galisson, Mahrouche et al. 2011).

We nevertheless continued focusing our attention on the functional implication of such mutation, rather than on its biochemistry.

***SUMO-dead* cells show a genomic instability signature.**

As previously mentioned, yeast cells carrying SUMO ligase-abrogating mutations in *mms21/nse2* show increased sensitivity to DNA cross-linking and UV irradiation, as well as a general chromosomal instability that commonly leads to gross genomic aberrancies (Bermudez-Lopez, Ceschia et al. 2010; Chen, Choi et al. 2009; Maria, Gangavarapu et al. 2007).

We tested for the presence of the same features in MEFs derived from *SUMO-dead* mice, and quantified their tendency to accumulate micronuclei and form polynucleated cells, either spontaneously or after the induction of DNA damage by MMS.

We devised such approach since micronucleation and polynucleation represent the pleiotropic outcome of different issues encountered by cells during replication or segregation - among which the accumulation of joint DNA molecules.

We observed that *Nsmce2*$^{SD}$ MEFs accumulated micronuclei spontaneously (**figure 16–A**) and that they generated *bi-nucleated* and *polynucleated* cells, possibly after aborting anaphase (**figure 16–B**).

Importantly, these phenotypes were in agreement with what observed after the impairment of dissolution, as in the case of BLM/Sgs1 mutant cells.

To further verify the dependency of these phenotypes on the amount of joint DNA molecules arising in replicating cells, we challenged replication by treating MEFs with MMS, failing to observe a proportional tendency to *micro-nucleation* or *poly-nucleation* in *Nsmce2*$^{SD}$ cells after treatment. We reasoned that such result could probably be due to a negative effect of the drug on cell proliferation, which consequently impeded the accumulation of replication-related by-products.

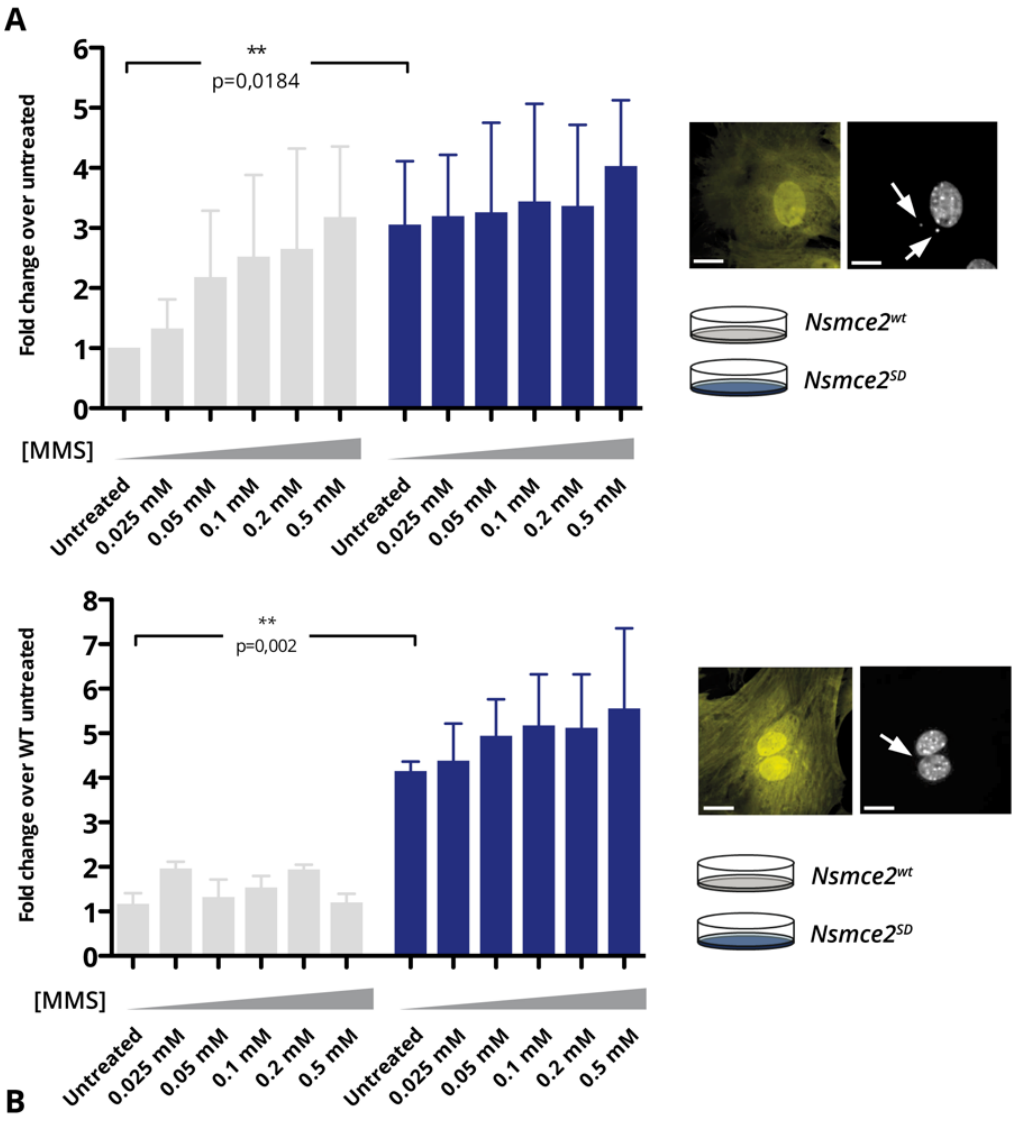



**Figure 16: *Nsmce2$^{SD}$* cells spontaneously accumulate micronuclei and show polynucleation.** Micronuclei accumulation and polynucleation was scored in SUMO-dead cells and wt counterparts (panel A and B, respectively). Sample pictures of scored events are represented on the right. Scale bar: 10μM**.**

To rule out that the SUMO-dead mutation might impair the assembly of the SMC5/6 complex, as well as alter the functionality of NSMCE2, we confirmed the preservation of the association between SMC5, SMC6 and NSMCE2$^{WT}$ or SD by immunoprecipitation and the ability of either NSMCE2$^{SD}$ and NSMCE2$^{WT}$ to form foci *in vitro* **(figure 17-A and B)**.

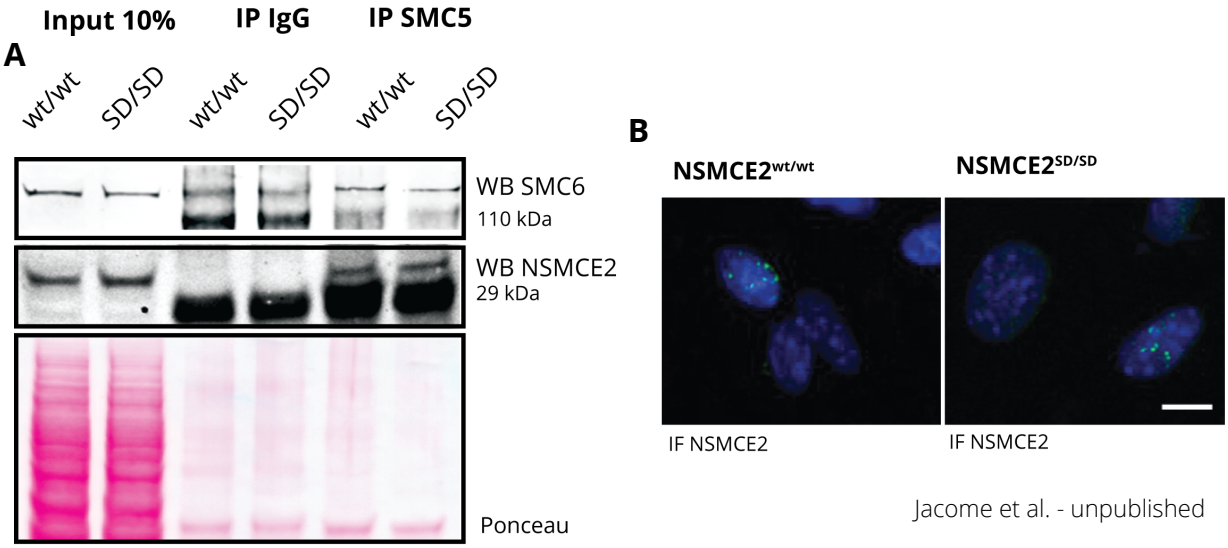



**Figure 17: The SUMO-dead mutation doesn't alter the composition of the SMC5/6 complex nor affect NSCME2 foci formation *in vitro.*** The SUMO-dead variant of NSMCE2 assembles in the SMC5/6 comparably to the WT counterpart, and is similarly able to form foci in vitro upon MMS treatment (10mM). Scale bar = 10 µm *(courtesy of A. Jacome).*

To further corroborate the genomic instability signature observed in fibroblasts, we shifted our attention to B lymphocytes and quantified the rates of sister chromatid exchanges (SCE) at mitosis, another major feature of BLM/Sgs1 mutant cells, as well as their tendency to spontaneous micronucleation and polynucleation.

We could indeed observe a significant increase of SCEs in *Nsmce2*$^{SD}$ B-cells, compared to their *wild-type* counterparts (**figure 18**), recapitulating, even if to a lesser extent, both the typical cellular hallmark of the Bloom syndrome (Chaganti, Schonberg et al. 1974) and the higher recombination rates observed for yeast *mms21* mutants (Prakash and Prakash 1977).

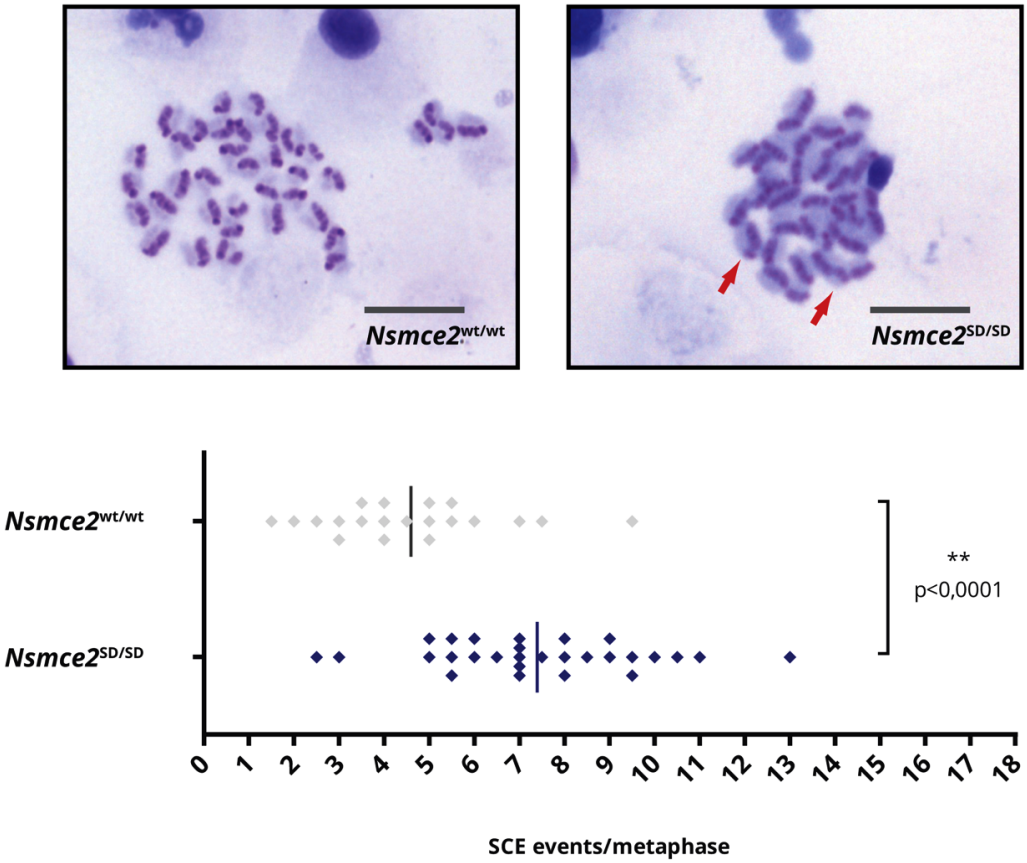



**Figure 18: lymphocytes from *Nsmce2$^{SD}$* animals show increased SCE rates**. Representative image of splenic B cells, obtained from WT or *SUMO-dead* animals and scored for the rate of SCEs upon nocodazole treatment. Scale bar: 15μm. The dot plot shows the quantification of SCE events/metaphases encountered in the samples.

### An intriguing hypothesis: NSMCE2 regulating topoisomerases activity?

Intriguingly, the increased recombination rates we observed *Nsmce2$^{SD}$* cells reminded of some of the phenotypes associated to the impairment of topoisomerases (Kegel, Betts-Lindroos et al. 2011; Olaharski, Mondrala et al. 2005). These enzymes prevent the intertwining that affects homologue DNA strands at the convergence of opposite replication forks, and they play an essential role in dealing with replication intermediates. As discussed earlier, in order to exert its function, BLM depends, in fact, on the physical interaction with topoisomerase IIIα (Hu, Beresten et al. 2001).

Both in yeasts and metazoans, mutations affecting topoisomerases are commonly associated to genomic instability (Bower, Karaca et al. 2010). Among the four subtypes encoded in the genome, class II topoisomerases play a pivotal role in mediating the disentanglement of joint DNA molecules before the onset of anaphase. By means of

several post-translational modifications, their activity is finely orchestrated with the replication machinery, and in such context SUMOylation exerts a fundamental function. (Ryu, Furuta et al. 2010). A possible NSMCE2-mediated regulation of topoisomerase activity hasn't so far been reported, but some literature underlines a genetic interplay between topoisomerases and the SMC5/6 complex (Harvey, Sheedy et al. 2004; Tapia-Alveal, Outwin et al. 2013). In order to investigate a possible role NSMCE2 in the regulation of topoisomerases, we took advantage of an *in vitro* DNA decatenation assay, allowing to probe for the activity of topoisomerase IIα. The results outlined in **figure 19** led us to conclude that, at least for the *in vitro* activity of topoisomerase IIα, the SUMO-ligase activity of NSMCE2 is dispensable.

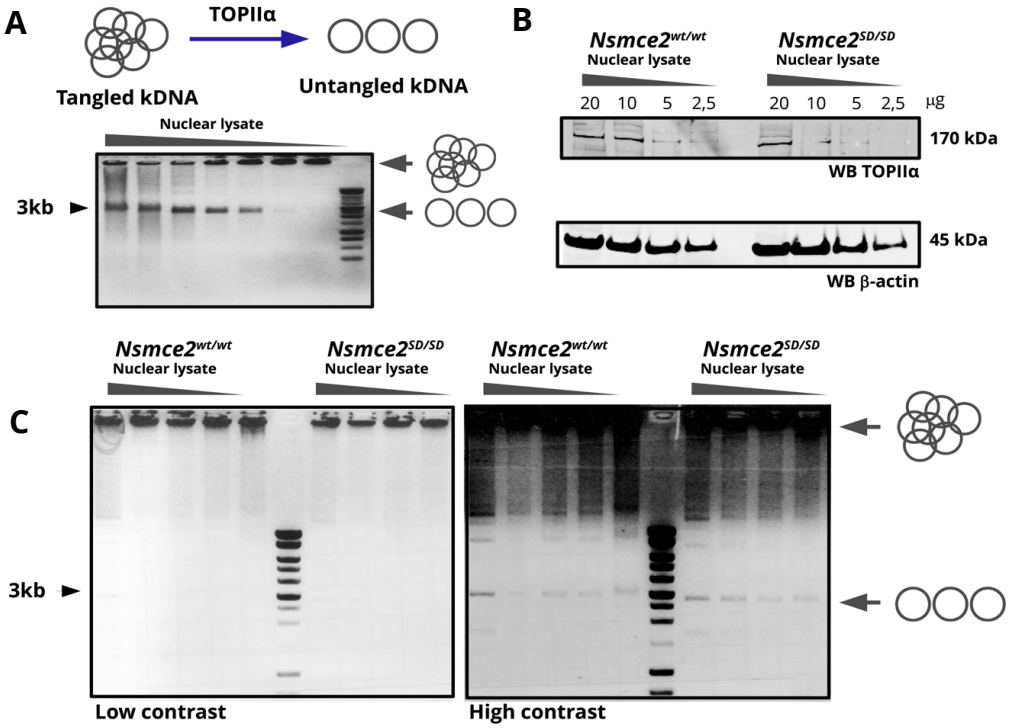



**Figure 19: NSMCE2 mediated SUMOylation doesn't affect TopIIα activity.** (A) TOPO activity was assayed *in vitro* by means of a kDNA disentanglement assay. (as in Material and Methods). (B) Purified nuclear extracts from wild-type and SUMO-dead cells were assayed for the levels of TopII α by WB. (C) TopIIα from both cell types can proficiently decatenate kDNA molecules.

Our observations didn't rule out, though, other possible regulations operated by the SMC5/6 complex in relation to topoisomerases. The complex may help targeting topoisomerases to their site of action, as an example. The budding yeast Smc5/6 complex was shown to localize to centromeres, telomeres and the chromosome arm

that harbours the ribosomal DNA arrays. All these are regions which are intrinsically prone to replication issues (Lindroos, Strom et al. 2006; Torres-Rosell 2005). Alternatively, other subtypes of topoisomerases could be the target of the SUMO regulation potentially operated by NSMCE2 (Kegel, Betts-Lindroos et al. 2011).

### *SUMO-dead* animals show hallmarks of genomic instability

To investigate the consequences of NSMCE2 SUMO ligase deficiency *in vivo*, we tested our *Nsmce2*$^{SD}$ mice for the presence of a genomic instability signature at the tissue level.

We first narrowed our search on the liver, a tissue that is particularly tolerant towards genomic aberrancies, and scored for the presence of either micronucleated or polynucleated cells in paraffin-embedded sections of the organ.

As reported in **figure 20**, *Nsmce2*$^{SD}$ animals accumulated more micronucleated and polynucleated cells than both their wild-type and *Nsmce2*$^{+/KO}$ heterozygous counterparts.

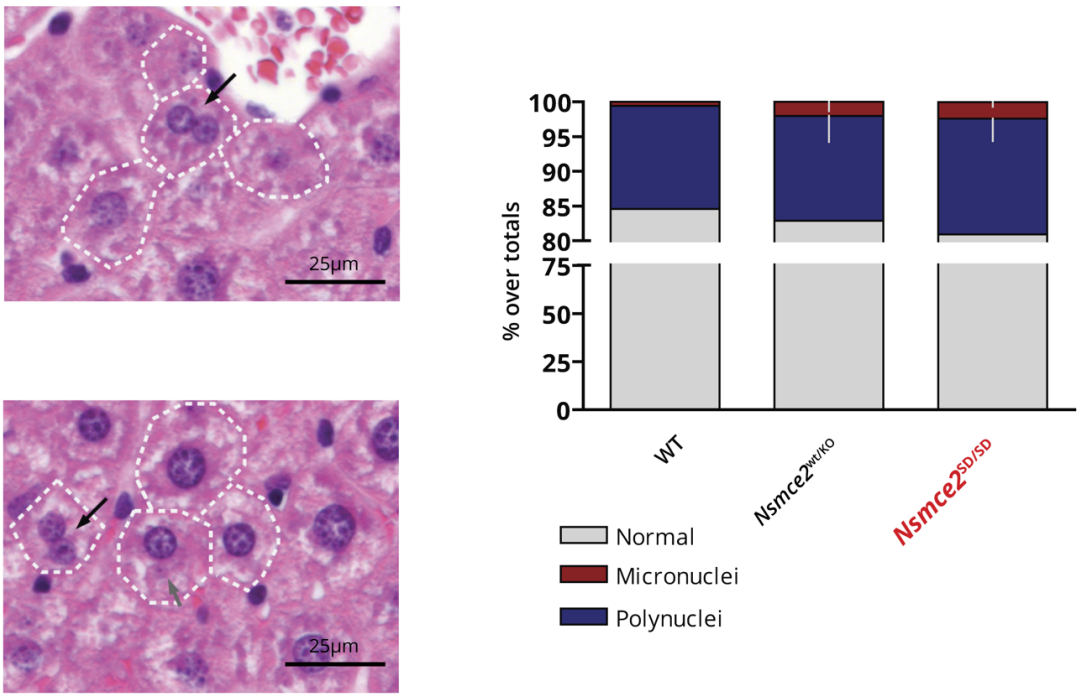



**Figure 20: *Nsmce2*$^{SD}$ animals show a systemic genomic instability signature.** Stained liver sections were analyzed for cells presenting polynucleation or accumulation of micronuclei. *Nsmce2*$^{SD}$ mice showed a divergent tendency to such accumulations when compared to controls. For every quantification, samples from n=4 animals were used.

To get further *in vivo* insights on the *genetic similarity* between NSMCE2 and BLM mutations, we decided to verify another typical hallmark of Bloom syndrome in our model. *Blm* mutations promote the insurgence of tumours and colon cancer is the most common single solid tumour in Bloom carriers (German, J. and Ellis, N. A. 2002). We interrogated our NSMCE2$^{SD}$ mouse model for the same tendency and performed an *azoxymethane-dextran sulphate* (AOM-DSS) induced carcinogenesis assay on a cohort of *wild-type* and mutant mice, following the methodology reported in *Nambiar, Girnun et al. 2003.*

We could highlight a modest, albeit statistically significant increase in the number of small-sized colon tumours in NSMCE2$^{SD}$ animals **(figure 21**), confirming our preliminary hypothesis and providing evidence supporting a "tumour protection" role for *Nsmce2*, at least in our experimental conditions.

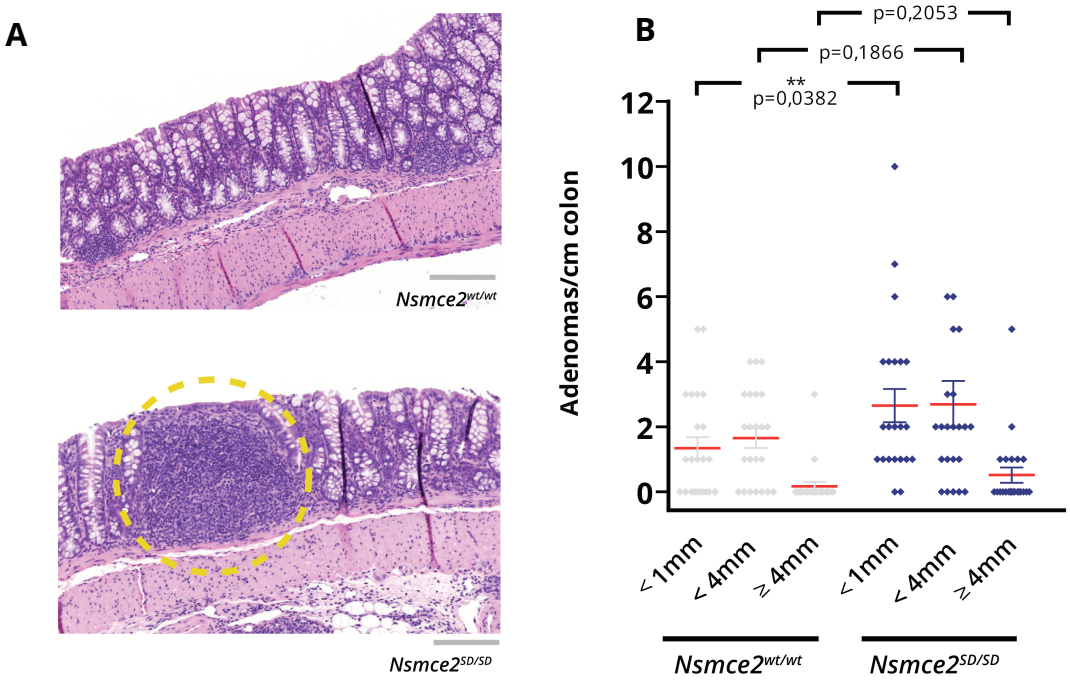



**Figure 21: *Nsmce2$^{SD}$* animals are prone to tumour formation.** (A) Representative image of paraffin-embedded sections of colon samples from *Nsmce2$^{wt}$* and *SUMO-dead* animals. A sample of scored tumours is circled in yellow. Scale bar = 200 µm. (B) *SUMO-dead* mice showed an increased tendency to develop colon tumours, recapitulating *in vivo* what observed in *Bloom* patients.

**The *Nsmce2$^{SD}$* mutation doesn't affect fitness and brings about a mild phenotype**

In order to further evaluate the physiological effect the *Nsmce2$^{SD}$* mutation, we analyzed the overall phenotype of animals and setup ageing curves of mutant animals against *wild-type* controls.

*Nsmce2$^{SD}$* animals were breeding normally and were undistinguishable from *wild-type* counterparts in terms or size and weight. (**figure 22-A**)

Unexpectedly, the mutation didn't affect the overall fitness of the *SUMO-dead* animals, as reported by the *Kaplan – Meier* curve in **figure 22-B**.

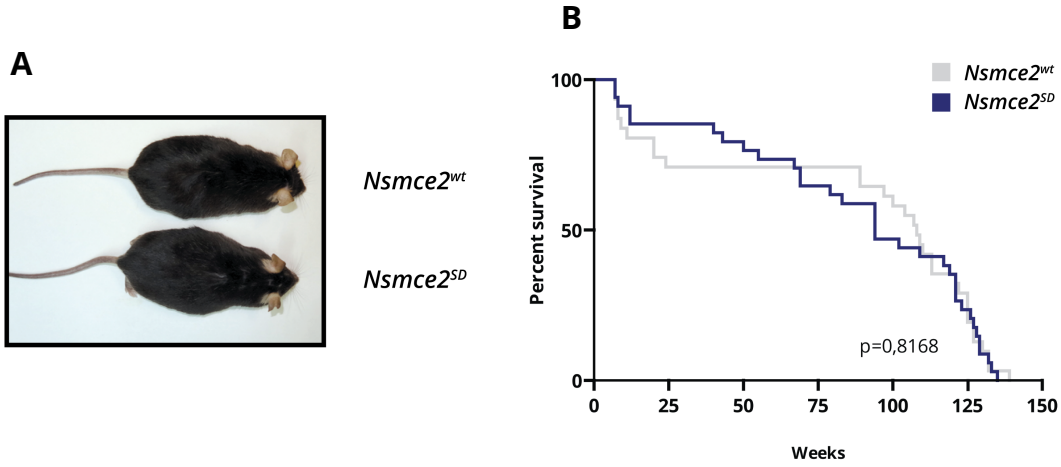



**Figure 22: the *Nsmce2$^{SD}$* mutation doesn't affect fitness.** Animals bearing the *Nsmce2$^{SD}$* mutation in homozygosis were undistinguishable from their WT counterparts. (panel A). We monitored a cohort of SUMO-dead animals/ (n= 35) and wild type controls (n=39) during roughly 3 years. The survival curve of *Nsmce2$^{SD}$* animals didn't diverge significantly from that of wild-type controls (panel B).

Their survival rate didn't indeed deviate from that of *wild type* animals, as well as the incidence of spontaneous tumours in these animals (*not shown*).

The mild phenotype of our *SUMO-dead* mouse model was unexpected. Despite the lack of a negative effect of survival, our observations *in vitro* (the increased rate of SCEs at mitosis, the increased proportion of micro nucleation and and polynucleation) as well as some *in vivo* hints (a mild accumulation of micronucleated and polynucleated cells in tissues, together with the increased tumorigenesis) recapitulated previous observations made in cells from Bloom patients.

Given the observed phenotypes, we decided to extend our analysis of the *Nsmce2$^{SD}$* allele in mice by investigating its genetic interaction with the resolution pathway.

## A genetic approach to frame the SMC5/6 complex in dissolution

### Intercross of *SUMO-dead* animals with a *Mus81$^{KO}$* strain

As mentioned, *Nsmce2$^{SD}$* mice bear some of features typically associated with genomic instability, at least at the cellular level. Considering that the observed phenotypes overlapped with what described for *Blm* mutants, we reasoned that a classical genetic approach might help elucidate the functional role of the SMC5/6 complex in dissolution.

In order to test such hypothesis, we decided to take advantage of mouse models for resolution described in the literature.

A first available candidate was the *Mus81 knock-out* mouse reported in *McPherson, Lemmers et al. 2004*. We rederived the strain on our mixed-background line, verifying at first the actual deficiency of the protein by Western blot. (**figure 23 - A**)

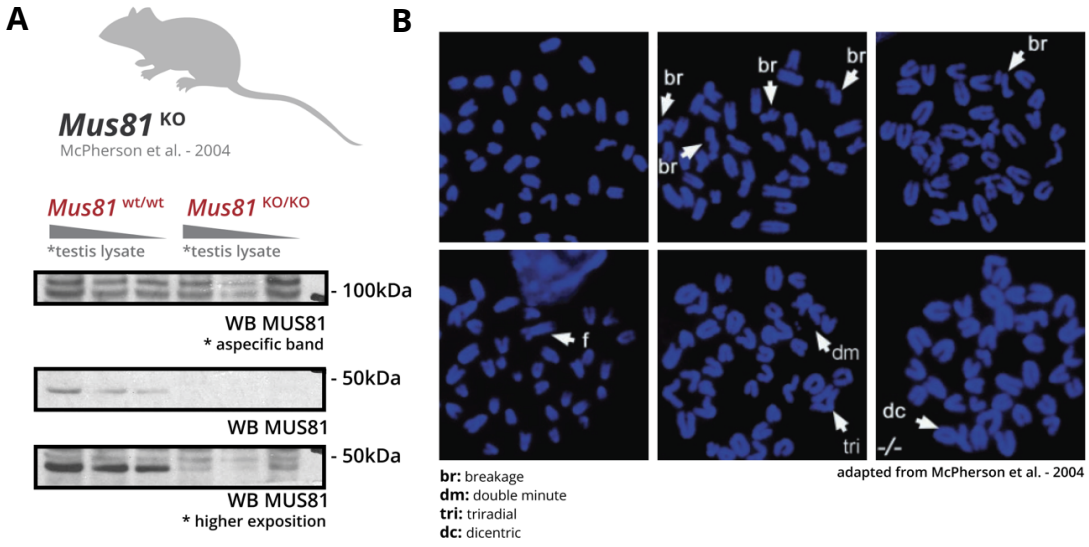



**Figure 23: *Mus81$^{KO}$* animals show a genomic instability signature.** (A) We could confirm the actual deletion of MUS81 in rederived animals by Western blot on testis protein samples. (B) A reported feature of *Mus81$^{KO}$* cells is a mild sensitivity to MMC. T-lymphocytes from *Mus81$^{KO}$* animals exposed to the drug accumulate chromosomal aberrancies as the ones highlighted by white arrows (adapted from *McPherson, Lemmers et al. 2004*)

Interestingly, the *Mus81$^{KO}$* strain we used was reported to show haplosufficient tumour suppression, which reflected in the survival rates of animals **(figure 24-A).** This striking result is in contrast with what observed in an alternative *Mus81-null* strain (Dendouga, Gao et al. 2005), which instead lacked any major phenotype **(figure 24-B),** and such

important contradiction warned us on the possibility of a background-related effect for the mutation.

Despite the remarkable survival discrepancy, both models concurred on a mild sensitivity to agents such as MMC, UV irradiation and MMS, as in **figure 25**.

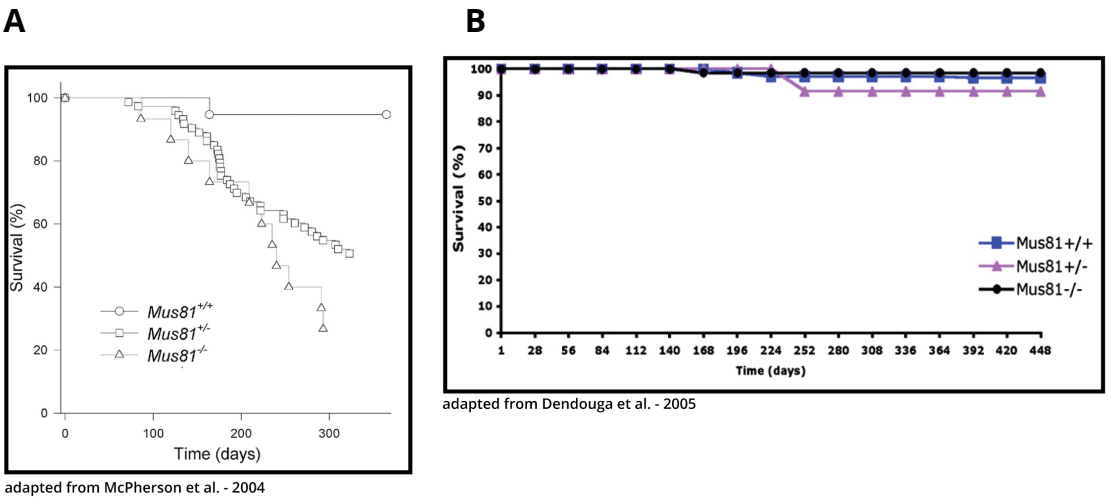



**Figure 24: discrepancies between *Mus81$^{KO}$* models available.** The two *Mus81-null* strains reported in the literature show a strikingly contradictory difference in terms of survival.(A) KM survival curve for the *Mus81$^{KO}$* model used in our study.(B) KM survival curve from the alternative *Mus81* model (McPherson et al. 2004; Dendouga et al. 2005).

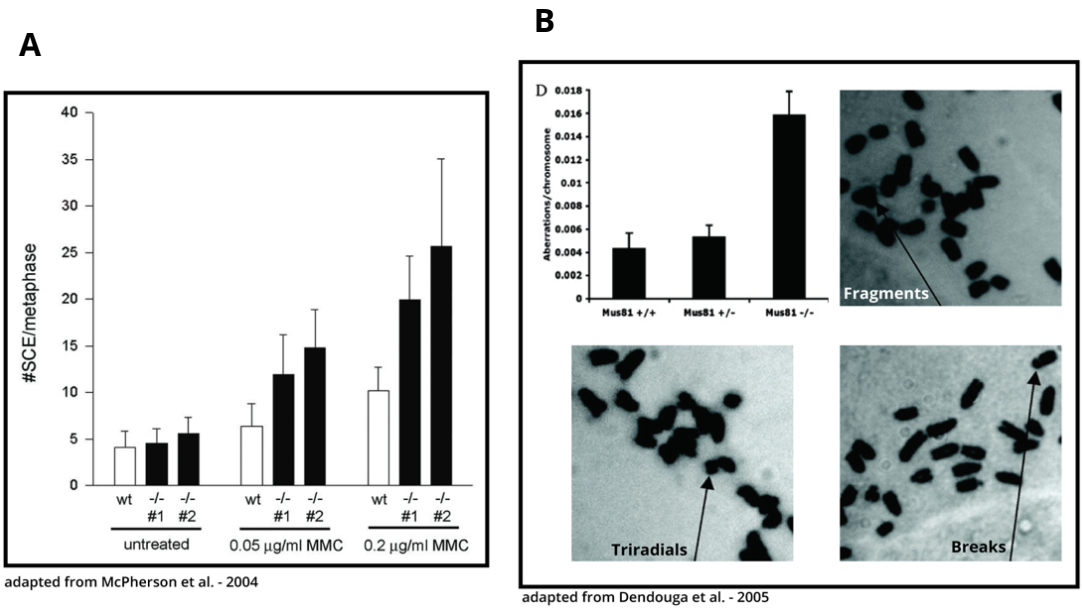



**Figure 25: all *Mus81$^{KO}$* strains show comparable sensitivity to DNA damaging agents.** Both *Mus81$^{KO}$* models respond consistently to DNA damaging drugs as highlighted by SCE experiments (A – McPherson et al. 2004) or metaphase spreads from B-cells (B – Dendouga et al. 2005)

Our first experimental approach aimed at verifying the outcome of impairing genetically both the resolution and the dissolution of DNA joint molecules, contemporarily. Numerous reports, as previously mentioned, show the lethal outcome of this genetic combination in lower eukaryotes. According to what first observed in yeast (Kaliraman, Mullen et al. 2001), *mus81* and *mms4* are also synthetic lethal with null mutations in *mus309*, which encodes the orthologue of the Bloom syndrome helicase in Drosophila (Trowbridge, McKim et al. 2007).

A possibility we consequently envisaged from our experimental setup was *synthetic sickness*: the inability to cope with replication intermediates would lead to the accumulation of joint DNA species and result in increased genomic instability, especially in organs and tissues with a sustained cellular turnover and high rates of cellular replication.

We thus continued by crossing our *Nsmce2$^{SD}$* animals onto the rederived *Mus81$^{KO}$* strain, defining a genetic scenario by which both resolution and dissolution (considering the genetic equivalence *Nsmce2 = Blm* we postulated) would be affected.

**The *Nsmce2$^{SD}$: Mus81$^{KO}$* mutations are not synergic in cells.**

The dissection of the possible genetic interaction between *Mus81* and *Nsmce2* followed a "simple to complex" approach. We started with experiments on MEFs derived from the *Mus81$^{KO}$* strain, reasoning that the outcome of impairing resolution could be a feedback up-regulation of the dissolution pathway, following a trend commonly observed in biological systems (Kitano 2004).

As a proof of principle, we quantified the accumulation of NSMCE2 foci in *Mus81$^{KO}$* cells, which could indicate an accumulation of joint DNA species demanding the activity of the SMC5/6 complex, as previously reported in our lab (*unpublished data*). We therefore performed NSMCE2 immunofluorescence stainings on *Mus81$^{KO}$* and ***wild-type*** cells, after treating them with a *sublethal* dose of MMS. As shown in **figure 26**, NSMCE2 did aggregate into bigger and more intensely stained foci upon the treatment with MMS, but no marked difference between the two lines could be emphasized.

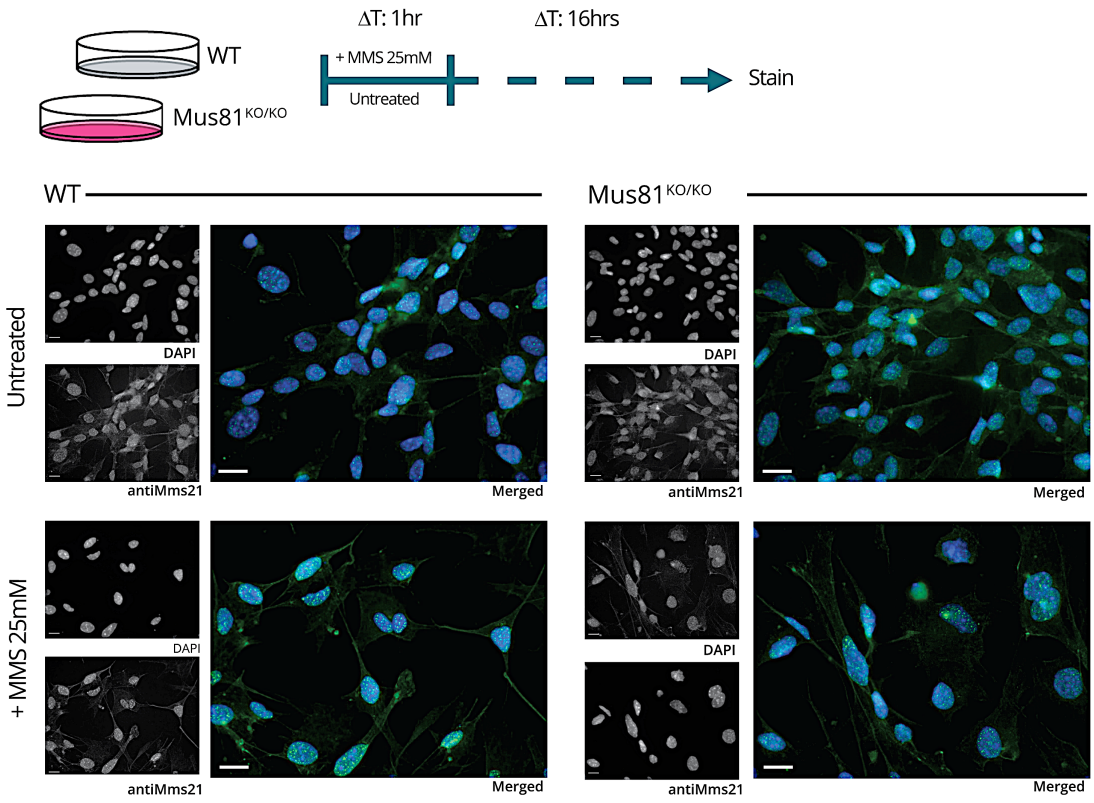



**Figure 26: NSMCE2 foci formation is unaffected by MUS81 depletion.** Primary fibroblasts from *Mus81*$^{KO}$ animals and wild-type controls were treated with MMS and and stained for NSMCE2/Mms21. Upon treatment, a similar accumulation of NSMCE2 foci was observed in both samples. Scale bar: 15µm.

These first negative observations don't rule out a possible functional interaction between NSMCE2 and MUS81. For that reason we sought additional evidence from the *Nsmce2*$^{SD}$ *: Mus81*$^{KO}$ double mutant model we had just generated.

As a first step, we verified that the protein levels of NSMCE2 wouldn't be affected by the deletion of *Mus81* (**figure 27**).

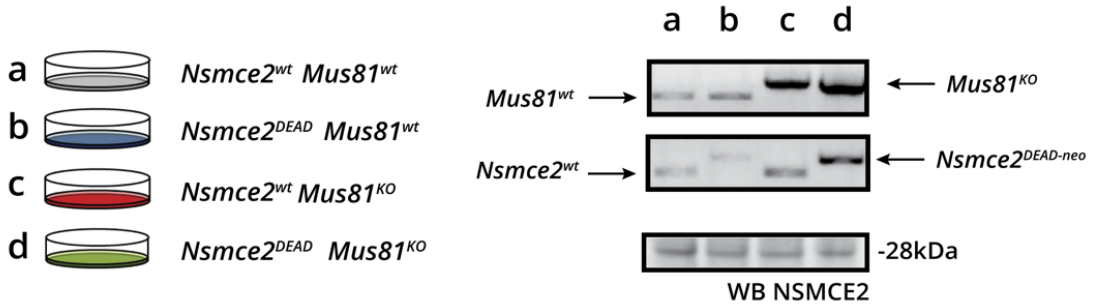



**Figure 27: NSMCE2 levels are unaltered after the deletion of *Mus81* in cells.** Cells from wild-type, *Nsmce2*$^{SD}$ *: Mus81*$^{KO}$ and relevant single-mutated controls were genotyped and blotted for NSMCE2. The levels of the protein were comparable to the wild-type controls in all genetic contexts.

We then proceeded along the line with what done for the *Nsmce2*$^{SD}$ model, and monitored the accumulation of micronuclei and polynucleation in MEFs obtained from *Nsmce2*$^{SD}$ *: Mus81*$^{KO}$ animals (and relevant controls).

As depicted in **figure 28**, *double-mutant* cells treated with increasing concentrations of MMS didn't respond differently from controls. We noticed a trend in the accumulation of micronuclei that followed the increasing doses of MMS treatment (at least in the case of *wild-type* cells) but in the other genetic backgrounds, the situation wasn't quite as defined.

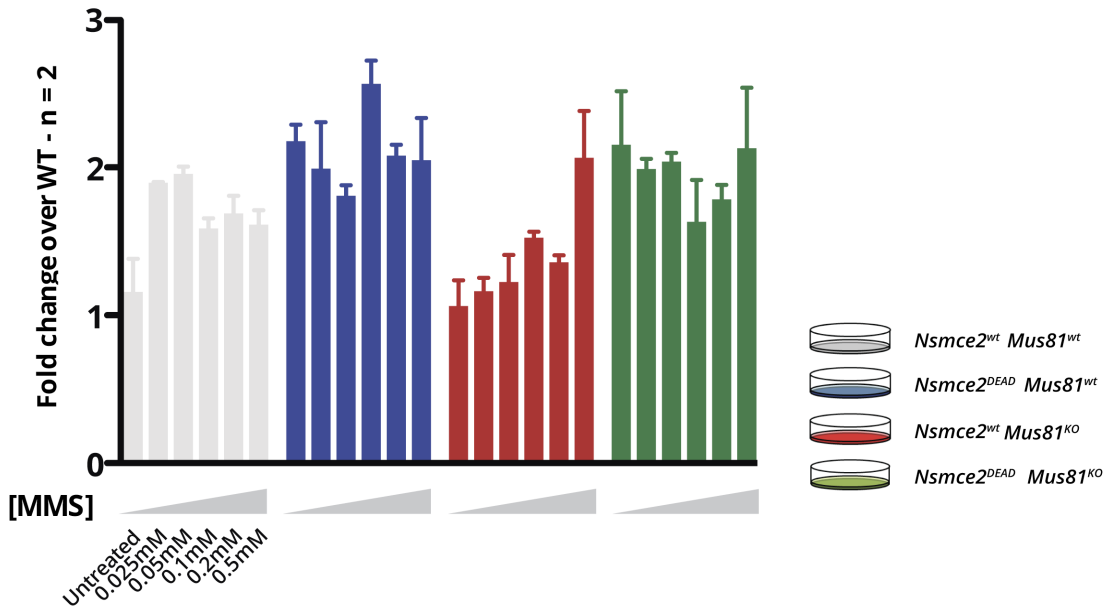



**Figure 28: *Nsmce2*$^{SD}$*:Mus81*$^{KO}$ show similar micronuclei accumulation as their control counterparts.** When scoring for micronuclei accumulation in the *double mutant* cell subtype, we couldn't highlight a negative synergy between mutated *Nsmce2* and *Mus81.*

*Mus81*$^{KO}$ cells, expectedly, accumulated less micronuclei than their counterparts, due to their sensitivity to MMS, an alkylating agent reported to impair cell proliferation at the concentrations we employed. (McPherson, Lemmers et al. 2004)

*Double mutants* behaved accordingly to what observed in the single mutants, inheriting the characteristics of both *Nsmce2*$^{SD}$ and *Mus81*$^{KO}$ alleles. They showed a basal tendency to instability, though such tendency couldn't be exacerbated by MMS treatment, probably because of the proliferation arrest driven by the absence of MUS81 in the presence of MMS, as previously reported (McPherson, Lemmers et al. 2004).

The quantification of polynucleated cells provided a similar outcome (**figure 29**): the basal levels of polynucleation in *single mutants* and *double mutants* were higher than wild-type controls, but the negative effect on proliferation impeded to highlight any incremental effect at increasing concentrations of the drug. The *double-mutants*, moreover, didn't show a worsened response than the *single mutants* alone.

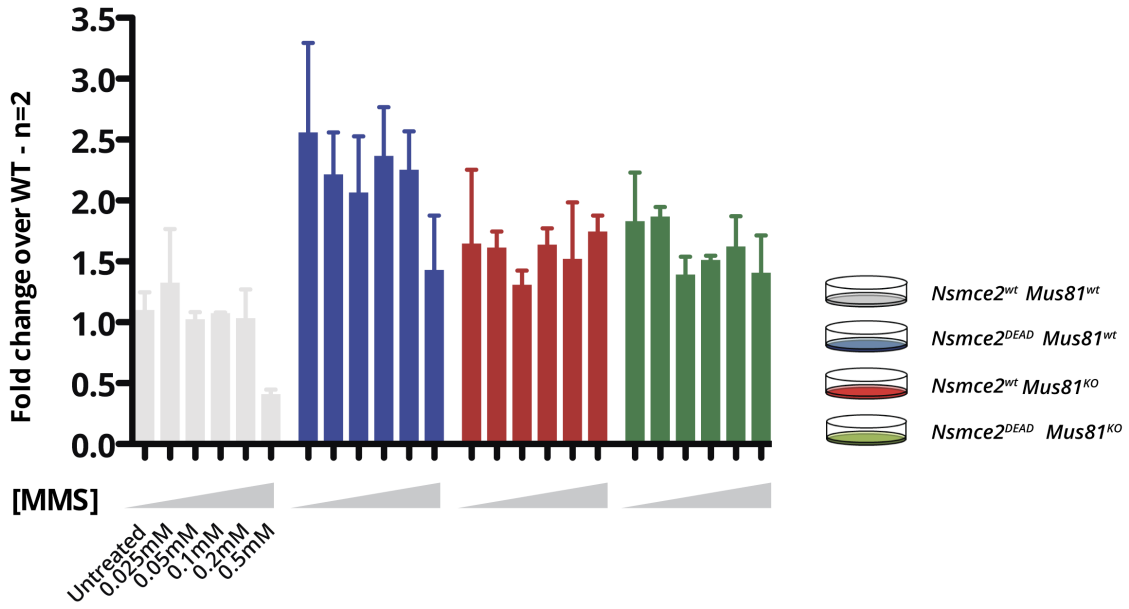



**Figure 29: *Nsmce2$^{SD}$:Mus81$^{KO}$* cells tend to polynucleation spontaneously, similarly to single mutant controls.** When scoring the polynucleation rates of *single mutants* and *double mutant* cells, we noticed a positive trend, but no direct correlation with the concentration of MMS used to induce damage.

To gain further insights into the DNA damage response of *Nsmce2-Mus81* double mutant cells, we analyzed the accumulation of nuclear 53BP1 foci upon treatment with damaging agents (such as MMC and MMS).

We envisioned that the depletion of MUS81 in a NSMCE2$^{SD}$ background - hence, in a context of accumulation of joint DNA molecules - could result in the breakage of unprocessed recombination intermediates at cell division. Consequently, the rupture of intertwined DNA molecules would promote DNA repair and the accumulation of 53BP1 at the site of breaks. We thus interrogated MEFs from our murine model for their propensity to accumulate 53BP1 foci, comparing double *Nsmce2$^{SD}$* - *Mus81$^{KO}$* mutants with their controls. In order to increase the accumulation of replication intermediates, we induced the collapse of replication forks by treating cells with increasing concentrations of MMC and a sublethal dose of MMS. The results outlined in **figure 30-A**

highlight how double mutant cells show an accumulation of 53BP1 foci comparable to that of single *Mus81*$^{KO}$ and *Nscmce2*$^{SD}$ mutants, at different MMC concentrations. Interestingly, the number of foci in both single and double mutant cells deviated from that of wt controls, suggesting, indirectly, the actual accumulation of broken DNA replication intermediates in these cells. As in the case of micro-nucleation and polynucleation though, no marked negative synergy could be detected.

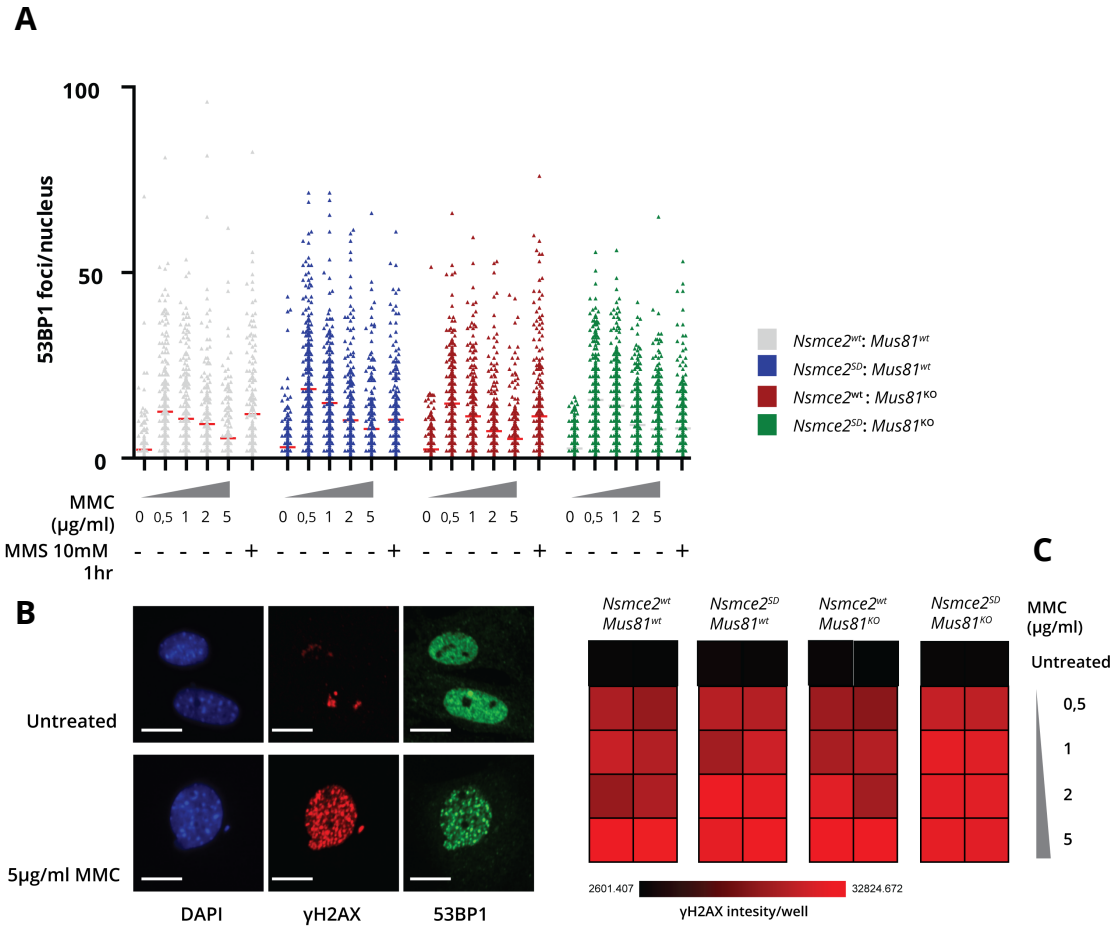



**Figure 30: *Nsmce2*$^{SD}$*-Mus81*$^{KO}$ cells show an unaltered response to DNA damage upon the induction of replication stress.** (A) 53BP1 foci were quantified by HT microscopy after the induction of replication stress (by means of MMC and MMS treatment) on double mutant cells and relevant controls. (B) Representative image of the induction of gH2AX and 53BP1 foci in cells upon treatment with MMC. Scale bar = 15 µm. (C) The accumulation of pan-nuclear gH2AX foci in cells (an established readout of replication stress) didn't differ markedly among the different lines.

Finally, we checked for the accumulation of chromosomal abnormalities in double *Nsmce2*$^{SD}$*-Mus81*$^{KO}$ cells and controls. Whereas we could detect the presence of aberrant chromosomal figures (such as radials, also commonly found in *Blm* cells, as in **figure 31** - circled in red) we didn't encounter a statistically significant difference between *wt* cells, *Nsmce2*$^{SD}$, *Mus81*$^{KO}$ or the double *Nsmce2*$^{SD}$- *Mus81*$^{KO}$ samples.

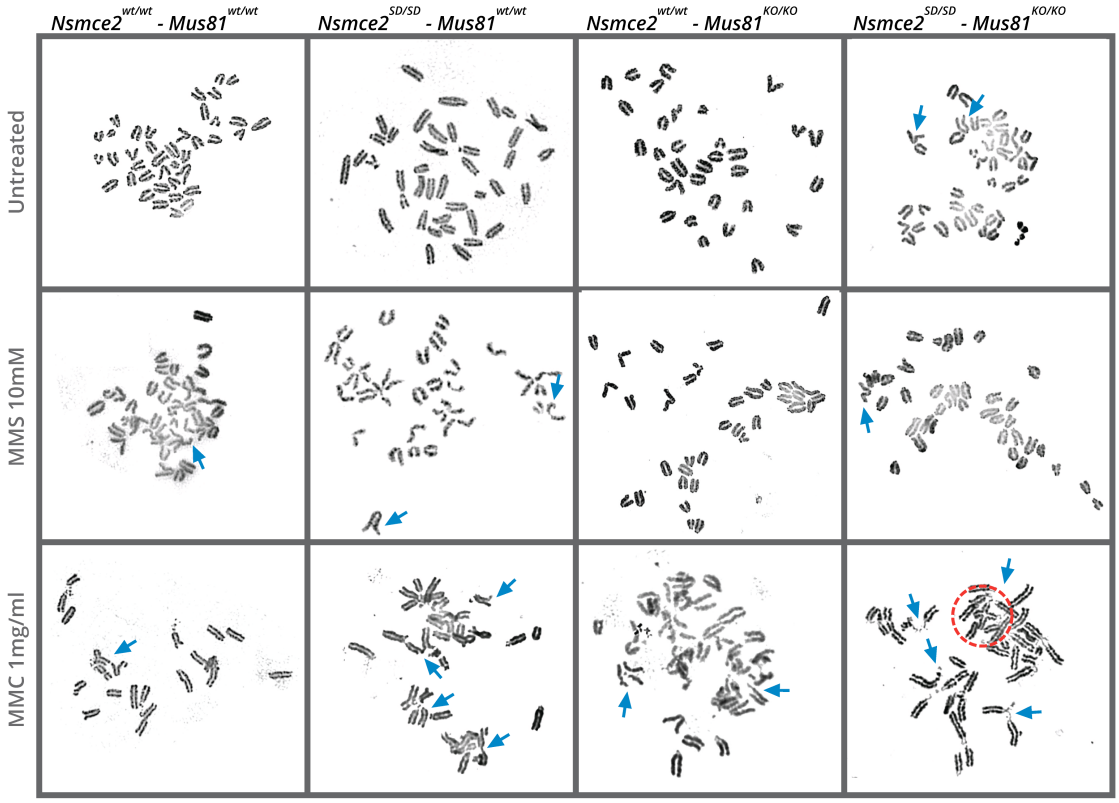



**Figure 31: the *Nsmce2$^{SD}$-Mus81$^{KO}$* mutations do not result in aberrant metaphases in MEFs.** Metaphase spreads were prepared from *wild type, Nsmce2$^{SD}$, Mus81$^{KO}$* and *Nsmce2$^{SD}$-Mus81$^{KO}$* cells, after treatment with either MMS or MMC, and chromosomal aberrancies were sought for (blue arrows). Though no striking effects were noticeable after MMS treatment, we highlighted the presence of radial chromosomes in MMC treated *Nsmce2$^{SD}$-Mus81$^{KO}$* cells.

### Compensatory effects of *Gen1-Slx1/4* in *Nsmce2$^{SD}$:Mus81$^{KO}$* cells?

It is possible that the lack of a clear phenotype in our cells could be due to compensatory pathways. The plausible functional redundancy of SLX1/SLX4 and GEN1 in mediating resolution could indeed counterbalance the absence of MUS81.

In order to shed a light on such possibility, we silenced, using RNAi, *Slx4* and *Gen1*, with the aim of impairing resolution of joint DNA molecules significantly.

Our experimental approach is summarized in **figure 32**.

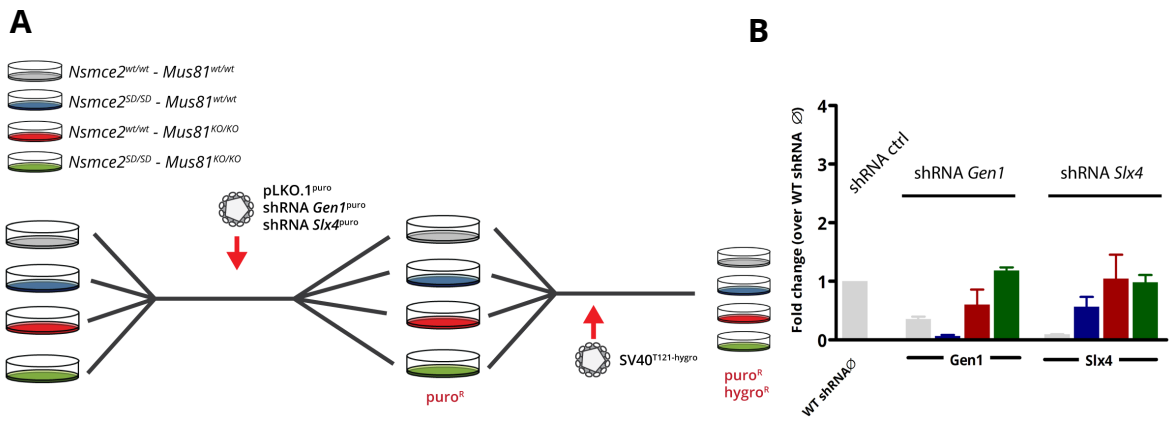



**Figure 32: experimental strategy to impair resolution in *Nsmce2$^{SD}$-Mus81$^{KO}$* cells.**(A) We used a lentiviral based platform to selectively silence *Slx4* and *Gen1* in double homozygous cells and controls with a *commercially validated* shRNA sequence. (B) After transduction and antibiotic selection, we measured the silencing of shRNA targets by RT-PCR, verifying their partial efficacy.

After transducing MEFs from our double mutant line and control strains with shRNAs against Gen1 and Slx4, we selected infected cells by adding puromycin to the culture medium.

Additionally, we immortalized them by infecting puromycin-selected pools with a retrovirally encoded SV40$^{T121}$, in order to overcome the possible replicative arrest of MEFs after transductions.

We tested for the efficacy of the shRNA mediated silencing by measuring the RNA levels of *Gen1* and *Slx4* in our treated cells (**figure 32-B**), verifying how only a partial *knock-down* of nucleases could be attained. Despite the mild silencing, we established growth curves of shRNA treated MEFs, either primary or T121 immortalized, to verify if the partial silencing of resolution nucleases could nevertheless result in impaired growth in a *Nsmce2*$^{SD}$-*Mus81*$^{KO}$ genetic background.

As in **figure 33**, all cell types responded aspecifically to the RNAi procedures, mostly by arresting proliferation (particularly in the case of Gen1 silencing). The same result was recapitulated when we tested both primary and immortalized MEFs for EdU incorporation (**figure 34**) Both the extensive manipulation and the partial functionality of the shRNAs employed (as well as plausible *off-targeting* effects of the shRNA employed) led to important effects on cell proliferation, jeopardizing the possibility of using this setup as an experimental platform.

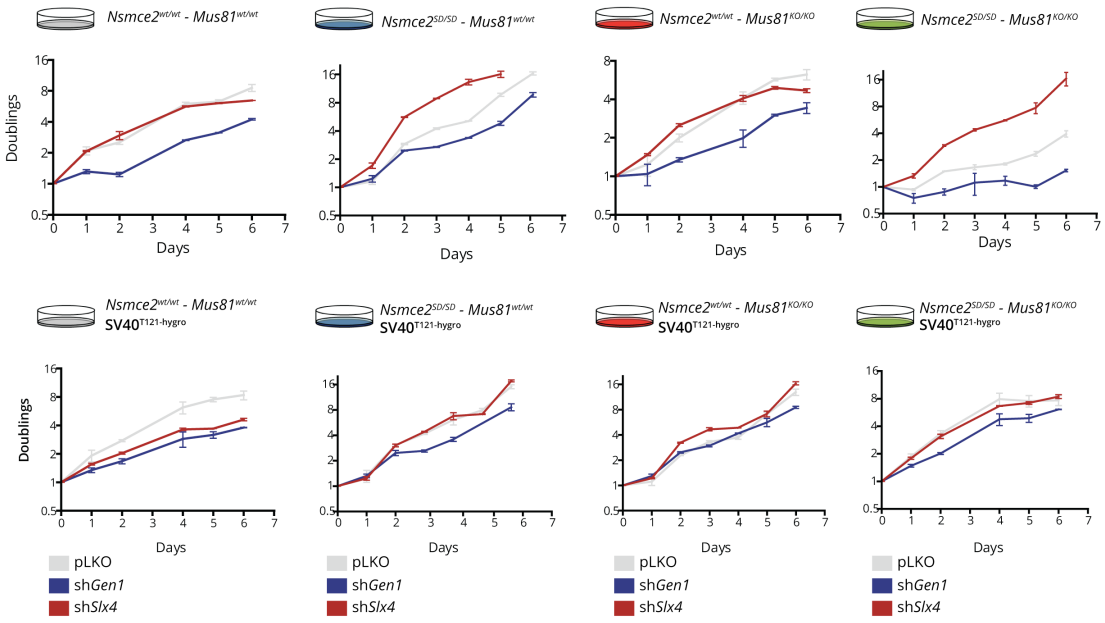



**Figure 33: the shRNA mediated silencing of endonucleases impairs cells proliferation aspecifically-#1.** Primary MEFs treated with shRNAs for *Slx4*, *Gen1* (or an empty control) tend to reduce their proliferation rate and enter proliferative arrest. Immortalization through SV40-T121recovers partially the phenomenon.

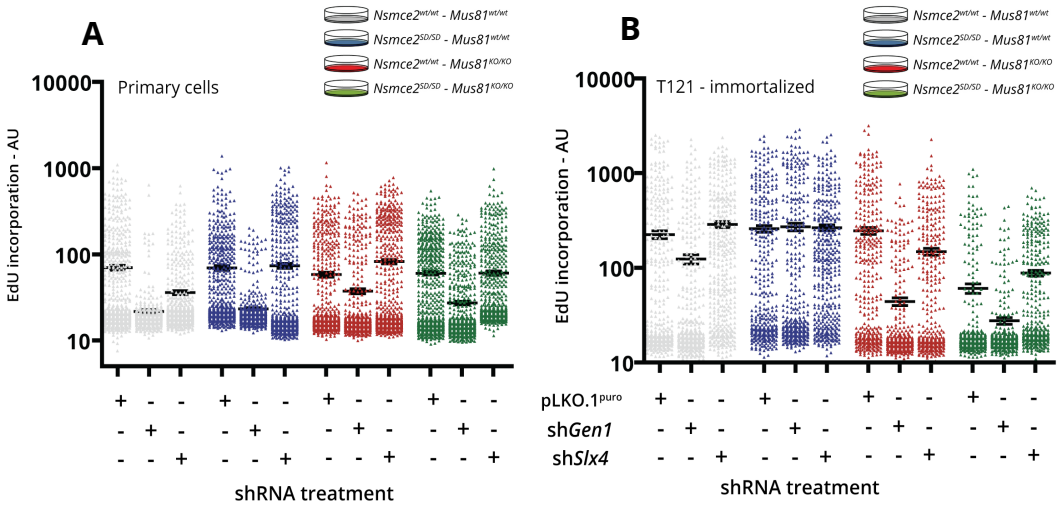
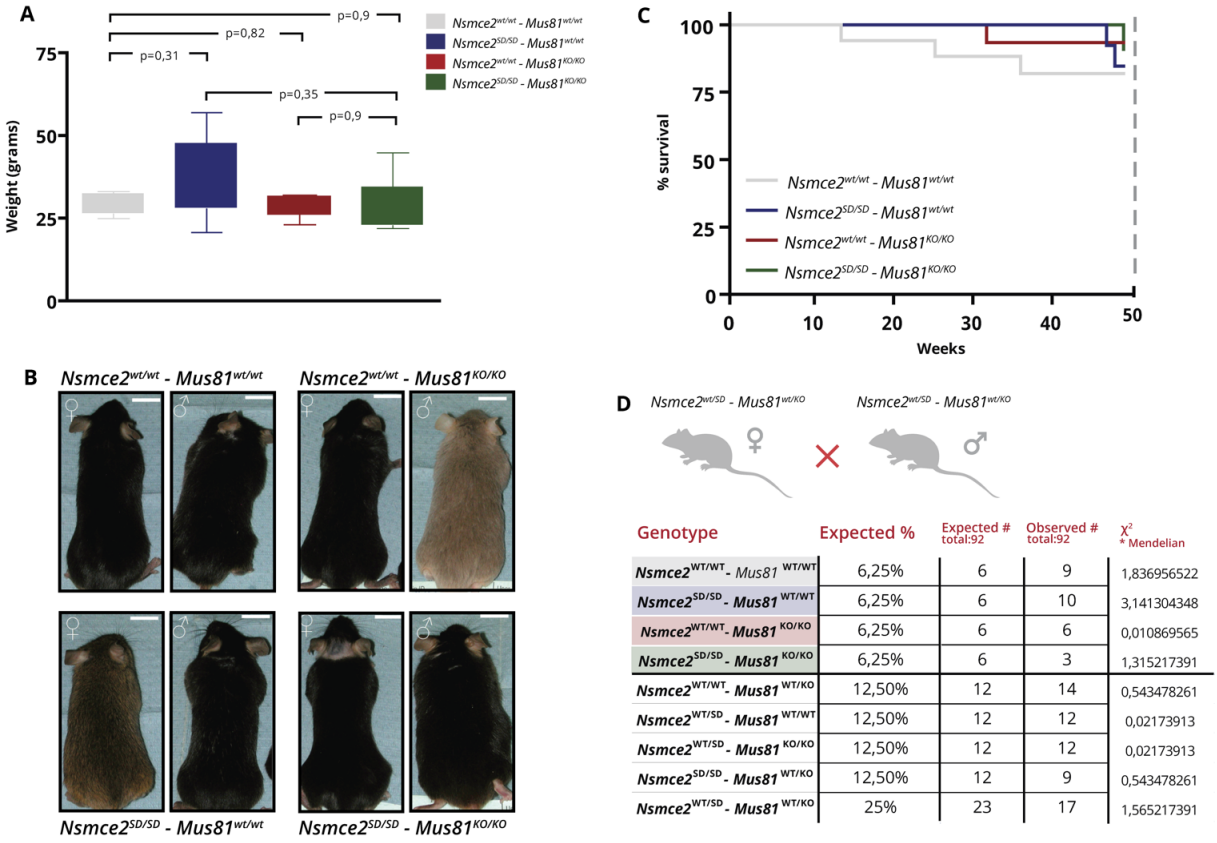



**Figure 34: the shRNA mediated silencing of endonucleases impairs cells proliferation aspecifically - #2.** Cells treated with shRNAs against resolution nucleases incorporate less EdU than controls. The negative effects on proliferation of shRNA treatments, confirmed by EdU incorporation experiments in primary MEFs (A), could only partially be recovered by SV40$^{T121}$ immortalization (B).

## *Nsmce2$^{SD}$ – Mus81$^{KO}$* animals don't differ significantly from control strains.

Despite the lack of a clear phenotype in MEFs, which suggested the lack of a synergic interaction between *Mus81* and *Nsmce2*, we analyzed the impact of the elimination of MUS81 on the *Nsmce2$^{SD}$* background *in vivo*. We hence focused on the *Nsmce2$^{SD}$-Mus81$^{KO}$* mice and setup ageing curves of double homozygous animals and relevant controls. At

the same time, we arranged for heterozygous crosses of *double heterozygous* mice, in order to verify the transmission of the *Mus81*$^{KO}$ and the *Nsmce2*$^{SD}$ alleles. As depicted in **figure 35**, no remarkable general differences between control animals and *Nsmce2*$^{SD}$ - *Mus81*$^{KO}$ double homozygous could be highlighted. Mice had a similar average weight, and their size wasn't significantly altered. The coexistence of both mutations, moreover, didn't affect the animals' fitness, since they survived as well as controls and bred normally. Finally, the allelic transmission of both mutant genes followed an expected Mendelian pattern, stressing the non-synergic effect of the two mutations, and the lack of *synthetic sickness*, despite our expectancy.




| Genotype | Expected % | Expected # total:92 | Observed # total:92 | $\chi^2$ * Mendelian |
|---|---|---|---|---|
| *Nsmce2*$^{WT/WT}$ - *Mus81* $^{WT/WT}$ | 6,25% | 6 | 9 | 1,836956522 |
| *Nsmce2*$^{SD/SD}$ - *Mus81* $^{WT/WT}$ | 6,25% | 6 | 10 | 3,141304348 |
| *Nsmce2*$^{WT/WT}$ - *Mus81* $^{KO/KO}$ | 6,25% | 6 | 6 | 0,010869565 |
| *Nsmce2*$^{SD/SD}$ - *Mus81* $^{KO/KO}$ | 6,25% | 6 | 3 | 1,315217391 |
| *Nsmce2*$^{WT/WT}$ - *Mus81* $^{WT/KO}$ | 12,50% | 12 | 14 | 0,543478261 |
| *Nsmce2*$^{WT/SD}$ - *Mus81* $^{WT/WT}$ | 12,50% | 12 | 12 | 0,02173913 |
| *Nsmce2*$^{WT/SD}$ - *Mus81* $^{KO/KO}$ | 12,50% | 12 | 12 | 0,02173913 |
| *Nsmce2*$^{SD/SD}$ - *Mus81* $^{WT/KO}$ | 12,50% | 12 | 9 | 0,543478261 |
| *Nsmce2*$^{WT/SD}$ - *Mus81* $^{WT/KO}$ | 25% | 23 | 17 | 1,565217391 |

**Figure 35: *Nsmce2*$^{SD}$*: Mus81*$^{KO}$ animals show no peculiar differences from control cohorts.** Neither their average weight nor size (as in A and B) was affected by the mutated alleles. (C) Animals survival rates where comparable, and the *Nsmce2/Mus81* alleles were transmitted at expected Mendelian ratios (D).

The normal phenotype arising from the genetic combination *Nsmce2*$^{SD}$ - *Mus81*$^{KO}$ came only partially as a surprise. On one side, our major concern was the lack of major consequences upon the depletion of MUS81, which is at odds with the striking phenotypes reported for the *Mus81*$^{KO}$ model we employed (McPherson, Lemmers et al.

2004). On the other side, though, the absence of a negative synergy between the two alleles further corroborated our observations on the mild phenotype presented by the *Nsmce2*$^{SD}$ strain.

These results prompted us to explore the involvement of the SMC5/6 complex in dissolution using other mutant strains of SMC5/6.

### *Smc6*$^{S994A}$ as an alternative genetic carrier.

As introduced previously, a major issue to study the SMC5/6 complex in mammals was the lack of appropriate murine working models. Taking in consideration the poor phenotype of *Nsmce2*$^{SD}$ *in vivo*, we opted for a recently characterized mutant for the SMC5/6 complex, presenting a stronger phenotype.

The strain bears a point mutation in *Smc6 (Smc6*$^{S994A\text{-}neo}$*)* (Ju, Wing et al. 2013) that recapitulates a separation of function mutation (*smc6*-S1045A) shown to confer increased genomic instability in *S. pombe* (Jessberger 2002) . It's to be underlined again how *Smc6* is an essential gene, both in yeast and metazoans (Ju et al. 2013) and that the outcomes of the *Smc6*$^{S994A\text{-}neo}$ mutation (an increased rate of SCE at mitosis and sensitivity to replication-challenging agents) *phenocopy* what observed in the case of several *mms21* mutants and functional equivalents (Doe, Murray et al. 1993; Lehmann, Walicka et al. 1995; Verkade, Bugg et al. 1999).

Apart from their genomic instability phenotype , *Smc6*$^{S994A\text{-}neo}$ homozygous animals are remarkably smaller in size, when compared to heterozygous and wt counterparts.

This allelic option represented hence a better experimental scenario for our purposes. By taking advantage of the *Smc6*$^{S994A\text{-}neo}$ strain, we could employ a mutant for the SMC5/6 showing similar *in vitro* features as *Nsmce2* mutants and possessing, moreover, a more evident and easily characterizable phenotype.

For this reason we rederived the strain on our mixed background and verified the preservation of the size anomaly reported for the model (**figure 36**).

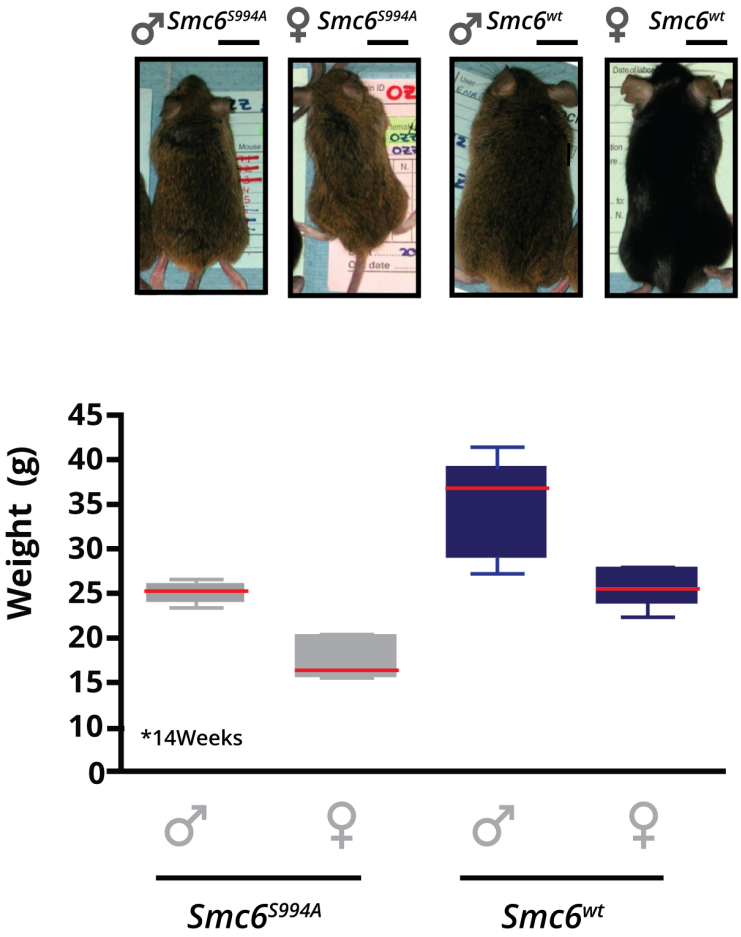
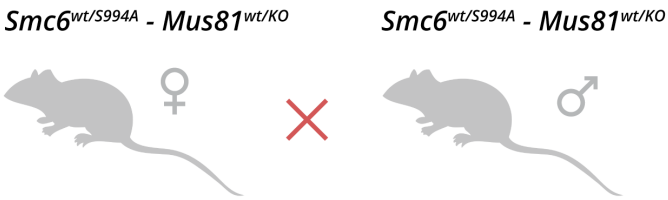



**Figure 36: *Smc6$^{S994A\text{-}neo}$* animals are smaller than wt counterparts**. We rederived the *Smc6$^{S994A\text{-}neo}$* mutation onto our mixed genetic background and verified the reduced size phenotype. The body size difference between adult (14 weeks) *Smc6$^{S994A\text{-}neo}$* animals and wt reflected their average weight difference.

According to the strategy outlined for the *Nsmce2$^{SD}$* strain, we crossed the new line with *Mus81$^{KO}$* animals, and, similarly to the previous case, established matings of heterozygous animals to verify the allelic transmission, as well as ageing curves of double homozygous mice and relevant controls. Strikingly, we encountered a strong *synthetic lethality* between *Smc6$^{S994A\text{-}neo}$* and *Mus81$^{KO}$*. We weren't indeed able to obtain neither double homozygous *Smc6$^{S994A\text{-}neo}$: Mus81$^{KO}$* animals out of the litters born of our experimental crosses (**figure 37**) nor double homozygous embryos, at least up to E10.5 *(data not shown).*




| Genotype | Expected % | Expected # total:137 | Observed # total:137 | $\chi^2$ * Non mendelian |
|---|---|---|---|---|
| *Smc6*$^{wt/wt}$ - *Mus81*$^{wt/wt}$ | 6,25% | 9 | 8 | 0,036953 |
| *Smc6*$^{S994A/S994A}$ - *Mus81*$^{wt/wt}$ | 6,25% | 9 | 6 | 0,76688 |
| *Smc6*$^{wt/wt}$ - *Mus81*$^{KO/KO}$ | 6,25% | 9 | 6 | 0,76688 |
| *Smc6*$^{S994A/S994A}$ - *Mus81*$^{KO/KO}$ | 6,25% | 9 | 0 | 8,5625 |
| *Smc6*$^{wt/wt}$ - *Mus81*$^{wt/KO}$ | 12,50% | 17 | 27 | 5,694343 |
| *Smc6*$^{wt/S994A}$ - *Mus81*$^{wt/wt}$ | 12,50% | 17 | 22 | 1,387774 |
| *Smc6*$^{wt/S994A}$ - *Mus81*$^{KO/KO}$ | 12,50% | 17 | 16 | 0,073905 |
| *Smc6*$^{S994A/S994A}$ - *Mus81*$^{wt/KO}$ | 12,50% | 17 | 4 | 10,05931 |
| *Smc6*$^{wt/S994A}$ - *Mus81*$^{wt/KO}$ | 25% | 34 | 48 | 5,520073 |

**Figure 37: *Smc6*$^{S994A\text{-}neo}$ and *Mus81*$^{KO}$ are synthetic lethal in mice.** The allelic transmission of *Mus81*$^{KO}$ and *Smc6*$^{S994A\text{-}neo}$ is non Mendelian. The same result was confirmed by the genotyping of embryos up to E10.5 (data not shown).

This striking result represented an obvious major drawback, for the impossibility to obtain animals or cells for experimental purposes. Interestingly though, despite the need for further characterization, the outcome of the *Smc6*$^{S994A\text{-}neo}$*: Mus81*$^{KO}$ intercross was supporting the hypothesis that impairing resolution on an "impaired SMC5/6 background" might severely affect fitness.

In order to overcome this issue, we devised to reproduce the same genetic combination by employing a *Gen1*$^{KO}$ strain we recently obtained (Matthews et al. *unpublished)* as an alternative strategy to affect resolution.

The *Gen1*$^{KO}$ mice we employed have no major phenotype, showing a normal lifespan and breeding normally *(*Matthews et al. *unpublished)*. Our new endeavour was frustrated, though, by the localization of *Gen1* and *Smc6* in the murine genome. Both genes co-localize on the same arm of chromosome 12 (**figure 38**) and their genetic linkage hinders their independent segregation, hence hampering the possibility of performing classical genetic analysis. We nevertheless believe that this finding is probably indicative of a functional relationship GEN1 - SMC5/6 complex.

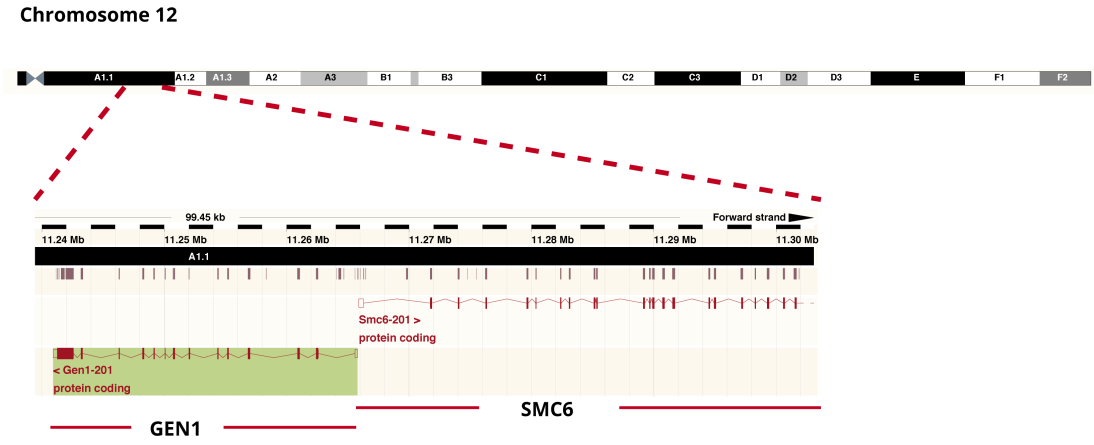



**Figure 38: *Smc6* and *Gen1* show genetic linkage.** We checked the localization of *Gen1* and *Smc6* in the murine genome through the ENSEMBL genome navigator (Flicek, Ahmed et al. 2013) verifying their close proximity, hence the impossibility to study their independent segregation. Intriguingly, this genetic linkage might suggest a potential genetic and functional interplay, as observed in the case of miRNA clusters (Altuvia, Landgraf et al. 2005).

## The quest for an alternative genetic carrier – the *Nsmce2$^{lox}$* strain.

The remarkable lethal interaction between *Smc6$^{S994A\text{-}neo}$* and *Mus81$^{KO}$* corroborated the hypothesis of a *functional bond* between the SMC5/6 complex and the metabolism of joint DNA molecules. The lack of homozygous animals to work experimentally, though, represented a major disadvantage. Therefore, in order to proceed, we crossed *Mus81$^{KO}$* mice with animals carrying a conditional allele of *Nsmce2* that we generated in the lab according to the strategy outlined in **figure 39** (Jacome et al. *unpublished*).

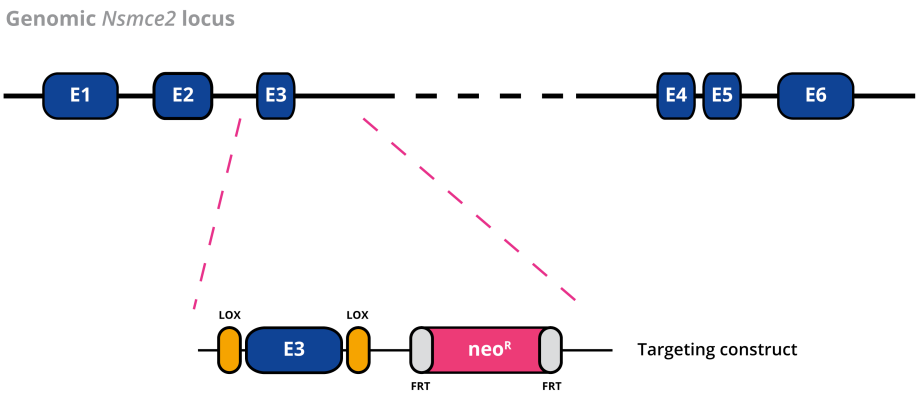



**Figure 39: Targeting strategy for the generation of the *Nsmce2$^{lox}$* strain.** ES cells from *wt* animals were targeted with a construct bearing the *floxed* exon3 of *Nsmce2* and a FRT-Neo$^{R}$ cassette for clonal selection purposes. Positive recombinants were microinjected in fertilized oocytes to generate the mouse strain employed.

*Nsmce2*$^{\Delta/\Delta}$ animals show an extended array of genomic instability features at the cellular level. Notably, cells depleted for NSMCE2 present a significant increase in SCE levels, (again, similarly to BLM-deficient cells), as well as a marked tendency to micro-nucleation. Additionally, *Nsmce2*$^{\Delta/\Delta}$ cells exposed to cross linking agents (such as MMS) leads to gross chromosomal aberrancies like the accumulation of radial chromosomes, recapitulating once more a molecular feature of *Blm* cells.

*In vivo*, the loss of NSMCE2 leads to a progressive and generalized progeroid syndrome, to which animals succumb within less than a year. *Nsmce2*$^{\Delta/\Delta}$ animals exhibit a number of pathologies which are also found in *Bloom's* patients, among which altered pigmentation, a reduced percentage of body fat, progressive anemia and a marked accumulation of micronuclei in cells with high proliferative turnovers such as the epithelium of the intestinal crypts (Lönn and Lönn 1990).

Considering our previous thwarted efforts when using fibroblasts, we decided to highlight any functional implications of the *Nsmce2*$^{\Delta/\Delta}$*-Mus81*$^{KO}$ genetic combination in B-lymphocytes, a paradigmatic cellular model where to study genomic instability for their tolerance to replication stress. In order to obtain experimental B cells, we intercrossed the newly generated strain with a Tg.CD19Cre line (Rickert, Roes et al. 1997), allowing for B-lineage restricted deletion of *Nsmce2*. Additionally, we devised to extend our analysis to embrace the different nucleases implicated in resolution and planned to ablate the whole set genetically. The absence of striking phenotypes observed *in vivo* upon the depletion of MUS81, as well as our results *in vitro* after the partial shRNA mediated silencing of *Gen1* and *Slx4,* were indeed supporting the possibility of a "functional takeover" by the different redundant nucleases implicated in resolution and were suggesting, at the same time, the need for a genetic approach, rather than a simple downregulation of the proteins.

We thus planned for crossing *Gen1*$^{KO}$ animals with conditional *Slx4*$^{lox}$ animals and *Nsmce2*$^{lox}$ conditionals, to finally generate a mixed *Slx4*$^{lox}$ *-Nsmce2*$^{lox}$ *- Gen*$^{KO}$ strain. This last model represents the most comprehensive genetic tool where to observe functional interactions between the SMC5/6 complex and the resolution machinery. SLX4, as previously mentioned, act as a docking platform regulating the activity of both MUS81 and SLX1, and its deletion hinders the activity of both partners. The conditional deletion

of SLX4 in our *Slx4*$^{lox}$ -*Nsmce2*$^{lox}$ - *Gen*$^{KO}$ strain would hence allow for a complete abrogation of the nucleases described to play a role in resolution. While the mice were being generated, we performed a first series of exploratory experiments on the *Nsmce2*$^{lox}$-*Mus81*$^{KO}$ line, still devoid of lineage-specific Cre. MEFs from *Nsmce2*$^{lox/lox}$–*Mus81*$^{KO/KO}$ animals, as well as single mutant and *wt* controls were used for these trials.

We ablated the *floxed* allele by transducing cells with a Cre-encoding adenovirus and after verifying the actual depletion of NSMCE2 (**figure 40**), cells were analyzed for highlights of genomic instability.

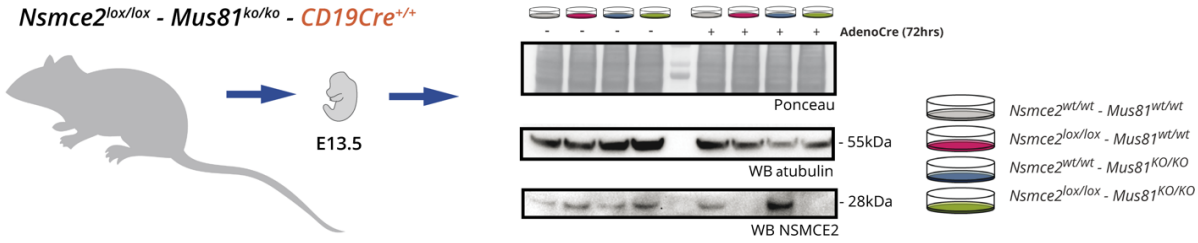



**Figure 40: The transient expression of Cre effectively ablates *Nsmce2* in MEFs.** Fibroblasts from E13.5 embryos were transduced with AdenoCre and the actual depletion of NSMCE2 was monitored 72hrs post infection (resulting in a virtually complete silencing).

As in our previous attempts, we employed these MEFs to verify their spontaneous tendency to genomic instability. Hence, we quantified their tendency to micronucleation and polynucleation, as in **figure 41**.

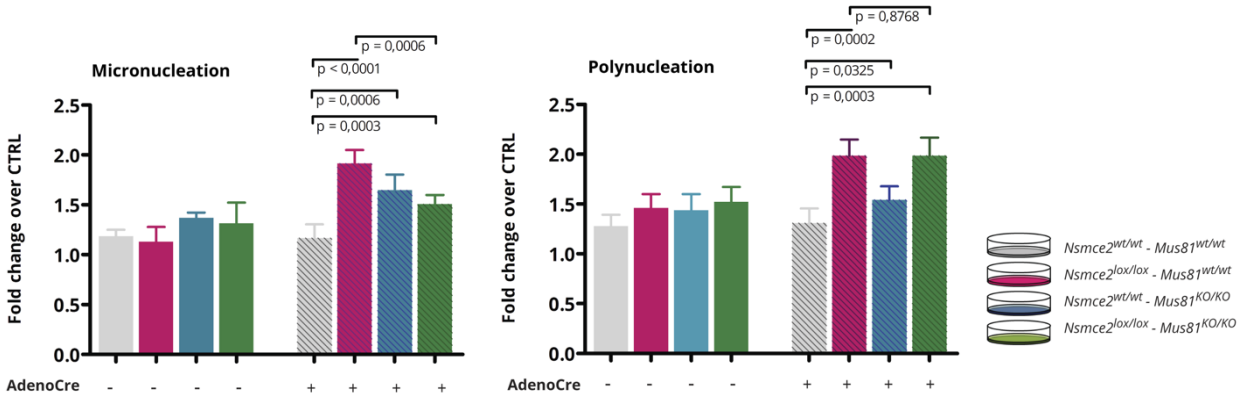



**Figure 41: Lack of a *phenotypical synergy* between *Nsmce2* and *Mus81* in MEFs from *Nsmce2*$^{lox}$-*Mus81*$^{KO}$ animals.** CRE-treated cells from the double homozygous animals are mildly prone to instability, but they seem to show no synergy between the detrimental effect of NSMCE2 ablation and MUS81 depletion.

### Deletion of *Mus81* in a *Nsmce2$^{\Delta/\Delta}$* background rescues increased recombination rates but doesn't grossly affect cell viability

A peculiar feature of *Blm* mutant cells, as previously mentioned, is the pronounced tendency to sister chromatid exchanges.

As outlined, the *Nsmce2$^{lox}$* mouse model recapitulates many of the features of *Blm* cells, in support of our original hypothesis of a genetic and functional equivalence *Blm* ≡ *Nsmce2*. Among them, comparable rates of spontaneous sister chromatid exchanges. As recently reported in human cells (Wechsler, Newman et al. 2011) such phenotype can be reverted, in *Blm* cells, by the concomitant depletion of MUS81. According to our model, the depletion of MUS81 in *Nsmce2$^{\Delta/\Delta}$* cells should result in the same reversal. We hence decided to verify the behaviour of *Nsmce2$^{\Delta/\Delta}$ - Mus81$^{KO}$* cells, performing SCE assays at mitosis with AdCre treated *Nsmce2$^{lox}$ - Mus81$^{KO}$*

Interestingly, we could indeed confirm what expected, as in **figure 42**.

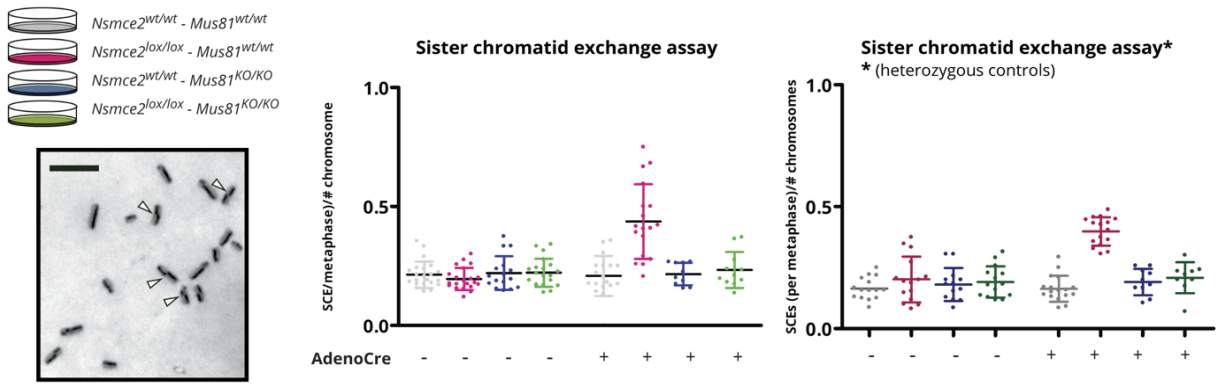



**Figure 42: The deletion of *Mus81* reverts the sister chromatid exchange phenotype observed in *Nsmce2$^{lox}$* cells.** Depleting NSMCE2 in replicating cells results in an increased rate of SCEs, as in the case of *Blm* (left panel). Similarly to what observed in *Blm* cells, MUS81 depletion in a *Nsmce2$^{\Delta/\Delta}$* background produces a reduction of sister chromatid exchange events (as quantified in the dot plots – right panel).

As in the previous cases, MEFs showed the a-specific effects of the experimental manipulation necessary to ablate *Nsmce2*. Though AdCre-treated *Nsmce2$^{lox}$* MEFs showed a mild tendency to accumulate micronuclei and to undergo polynucleation, we failed to highlight a synergy between then concomitant depletion of NSMCE2 and MUS81, presumably due to the aspecific proliferative arrest induce on fibroblasts by the AdCre transduction.

The detrimental effect of AdCre on cell proliferation was confirmed in the colony forming assays (CFAs) performed on *Nsmce2*$^{lox/lox}$ – *Mus81*$^{KO/KO}$ cells and relevant controls (**figure 43**).

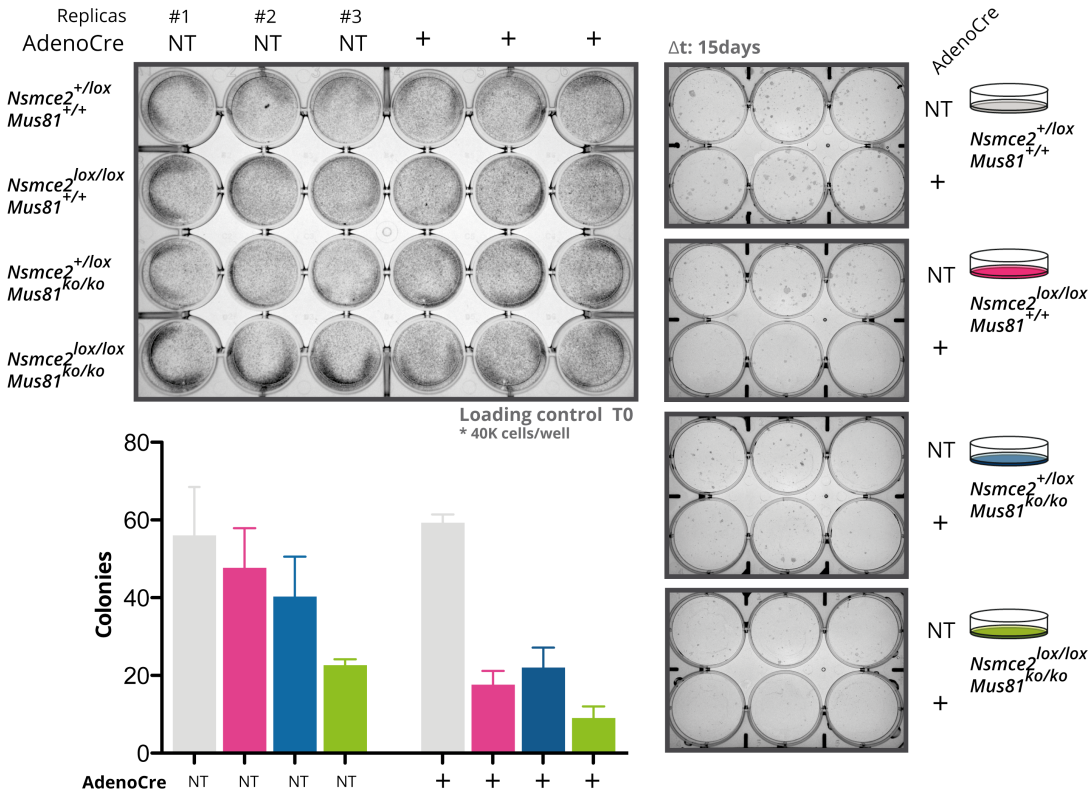



**Figure 43: *Nsmce2*$^{\Delta/\Delta}$*: Mus81*$^{KO}$ MEFs show similar growth disadvantages to *Nsmce2*$^{\Delta/\Delta}$ and *Mus81*$^{KO}$ cells.** Primary MEFs were extracted at E13.5, immortalized by SV40$^{T121}$ and transduced with AdCre and plated to test survival and colony formation Surviving colonies were scored after 15 days. In addition to a non-specific toxic effect of the immortalization procedure and the detrimental effect of Cre transduction, which are evident in all but *wt* cells, double homozygous cells didn't show a stronger growth disadvantage than control counterparts upon NSMCE2 depletion.

As evident from the results of the CFAs, genetically modified cells transduced with the AdenoCre virus underwent proliferation arrest, and such tendency was particularly evident in the case of *Mus81*$^{KO}$ cells. This response was once more jeopardizing the possibility to quantify the effects of our genetic background on cell survival in MEFs, and was suggesting to focus on an alternative cellular model.

We thus analyzed B-lymphocytes obtained from our *Nsmce2*$^{lox}$*-Mus81*$^{KO}$*-CD19Cre* model, searching for instability phenotypes that could corroborate the function interplay *Mus81-Nsmce2*.

For that purpose, we extracted cells from the spleen of experimental animals and checked for the effective *cleavage* on *Nsmce2* operated by the endogenous Cre, verifying its efficacy (**figure 44**).

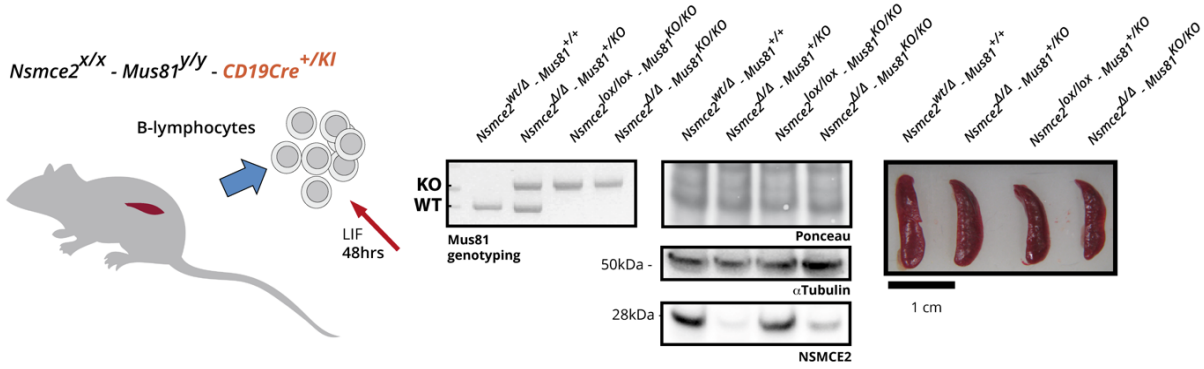



**Figure 44: Effective depletion of NSMCE2 in *Nsmce2$^{lox}$-Mus81$^{KO}$-CD19Cre* animals.** The CD19Cre used in our strain effectively process *Nsmce2* in B-cells, as highlighted by Western blot. As in the right panel, organs obtained from double homozygous animals didn't show dysplasia nor differ macroscopically from the ones of control animals.

According to our preliminary hypothesis, a mutant for *Nsmce2* and *Mus81* should show the negative effects of the excessive accumulation of joint DNA replication intermediates.

We were reasoning, as previously underlined, that the concomitant impairment resolution (resulting from the deletion of Mus81) and dissolution (originating from the ablation of *Nsmce2*) would result in synthetic sickness, producing an array of detrimental consequences in actively replicating cells. We thus first analyzed B-lymphocytes from our animals macroscopically, and focused on parameters such as cellular morphology and size. Genomic aberrations like aneuploidy and gross chromosomal rearrangements commonly reflect in alteration of nuclear morphology (Gisselsson, Björk et al. 2001). In the case of B-cells, nuclear abnormalities would obviously result in cellular dysmorphism, due to the prominent size of their nucleus. Interestingly, in agreement with this, the concomitant deletion of *Nsmce2* and *Blm* in B-cells leads to the formation of multilobulated nuclei (Jacome et al. *unpublished*)

We monitored the proliferation rates of *Nsmce2$^{lox}$-Mus81$^{KO}$-CD19Cre* cells, taking into account that deregulated proliferation is one of the most clinically relevant readouts of

genomic instability. Once again, despite our expectations, no clear difference was noticeable among different samples.

As in **figure 45-A**, no macroscopic difference was evident between cells derived from experimental animals and controls. The gross morphology of *Nsmce2$^{lox}$-Mus81$^{KO}$-CD19Cre* and relevant controls wasn't differing, as suggested from the SSC and FSC of cells samples analyzed by FACS (**figure 45-B**). At the molecular level, we could not highlight major chromosomal aberrancies, either (**figure 45 panel B - right**).

Intriguingly, we could once again verify, in this alternative cell type, the "reversal phenotype" on the rate of sister chromatid exchanges that we observed in MEFs after the concomitant deletion of *Nscme2* and *Mus81* (**figure 45-C**).

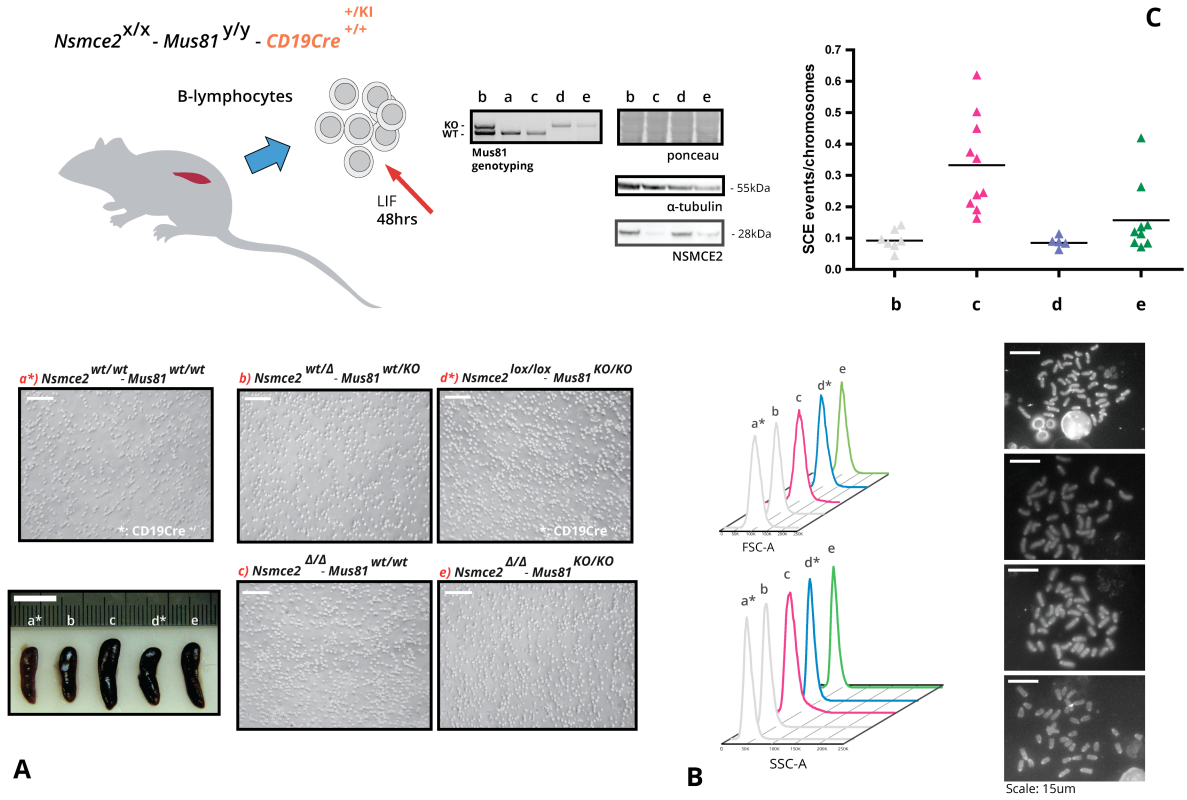



**Figure 45: B-cells from *Nsmce2$^{Δ/Δ}$-Mus81$^{KO}$* animals don't show relevant abnormalities.** As in panel A, cells from control animals and *Nsmce2$^{lox}$-Mus81$^{KO}$ – CD19Cre* mice don't show major difference in terms of size and morphology. In confirmation, their FSC and SSC profiles are undistinguishable (panel B). When checking sampled metaphases from *Nsmce2$^{Δ/Δ}$ : Mus81$^{KO}$* cells, moreover, no striking features could be underlined. (panel B - scale bar = 10μm). Despite the lack of a clear macroscopic difference, we could notice, as in MEFs previously, the reversal of the SCE phenotype proper of *Nsmce2$^{Δ/Δ}$* cells, upon the concomitant deletion of *Mus81* (panel C).

This last result, confirmed in two independent cell types, was suggesting the direct implication of MUS81 in the processing of DNA replication intermediates originating from the impairment of the SMC5/6 complex. While this, as previously mentioned, agreed with what reported for BLM deficient cells (Wechsler, Newman et al. 2011), it was at odds with the lack of negative effects on the overall health of cells. Several possible explanations may account for that. This could be reasonably due to cell specific effects, primarily. The cell types we analyzed (MEFs and B-cells), which are routinely used in the field for their relative tolerance to DNA damage, may represent the wrong cellular *milieu* where to study the interplay between the SMC5/6 complex and the resolution machinery. We can't indeed rule out *a priori* the existence of uncharacterized regulatory mechanisms that may interfere with our analysis.

Additionally, we can't exclude a selective processing of different subsets of joint DNA molecules. MUS81 could be specifically implicated in the recombinogenic resolution of joint DNA species. SLX1/4 and GEN1 would instead catalyze non recombinogenic resolution, preventing the accumulation of SMC5/6 related DNA intermediates (and their detrimental effects) in the cells we analyzed.According to this hypothesis, the depletion of MUS81 in a SMC5/6 background would only result in the prevention of sister chromatid exchanges, without major consequences in terms of cellular health, as we effectively observed.

In conclusion, we esteem that further experiments on the $Slx4^{lox}$ -$Nsmce2^{lox}$ - $Gen^{KO}$ strain being currently generated in the lab may help shed a light on the actual role of the SMC5/6 in the metabolism of joint DNA molecules.

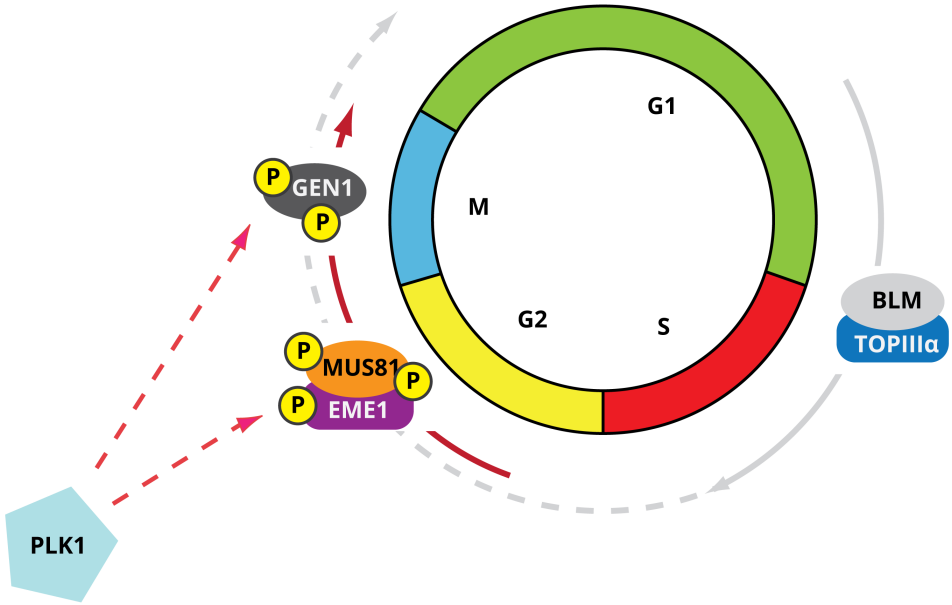
# Results – Part II

# Results - part II

## Molecular characterization of the resolution nucleases

While we concentrated our efforts on working genetically to define the interplay between the SMC5-6 complex with the dissolution and resolution machinery, we decided to explore the molecular insights of resolution, which are still vastly uncharacterized in higher eukaryotes.

Our endeavours were inspired by the current regulatory model proposed for BLM/TOPIIIα, MUS81 and GEN1 (Matos, Blanco et al. 2011). According to the latter, BLM/TOPIIIα would operate the dissolution of DNA joint molecules all along the cell cycle, and exert its function, primarily, before the onset of the M-phase. After the entry in G2, activated Polo kinase(s) would operate a series of "licensing phosphorylations" on specific Ser/Thr residues of GEN1 and MUS81/EME1/Mms4, activating them and promoting the resolution of DNA replication intermediates that might have accumulated after the completion of the S-phase. (**figure 46**).




Adapted from *Matos el al. - 2011*

**Figure 46: cell cycle regulation of the activity of dissolution and resolution.** According to the current consensus, BLM-TOPIIIα would operate a background monitoring of the accumulation of DNA replication intermediate all along the cell cycle. By operating phosphorylation on Ser-Thr residues of GEN1 and MUS81-EME1, PLK1 activates the enzymes, promoting resolution post-S phase (adapted from Matos, Blanco et al. 2011)

Interestingly, in agreement with the model, premature activation of Mus81 in yeast, achieved with phosphomimetic Mms4 variants, was shown to induce crossover-associated chromosome translocations (Szakal and Branzei 2013).

In summary, the dissolution machinery would operate a “background monitoring” preventing the accumulation of DNA joint molecules all along the cell cycle. The activation of endonucleases in late G2/M would represent a safeguard mechanism to avoid the persistency of DNA replication intermediates during mitosis. By resolving joint DNA molecules right before cell division, GEN1 and MUS81 would promote the correct segregation of chromosomes at anaphase, at the cost, though, of an increased probability of unbalanced sister chromatid exchanges and potential LOH.

Taken into account such tight regulation, and the observation from yeast experiments mentioned above, we questioned ourselves on what could happen if nucleases were to be activated in the “wrong” phase of the cell cycle in higher eukaryotes.

A plausible possibility could be that prematurely activated enzymes would operated an extensive processing of DNA replication intermediates accumulated during replication, giving rise to massive DNA damage and finally wreaking havoc on the cellular genome.

### Chromosome pulverization: a role for structure specific nucleases?

We encountered a potential and intriguing parallelism to our question in some early work from the late '60, where an interesting phenomenon – *chromosome pulverization* – was reported in detail (Kato and Sandberg 1968, Matsui, Weinfeld et al. 1971). A number of groups almost concomitantly described the outcome of experiments where a cell in interphase was fused with a metaphase cell (Obe, Ludcke et al. 1975; Rao and Wilson 1976). In such scenario, an unknown agent from the metaphase nucleus could induce the *heterochromatization* of chromosomes, either in their unreplicated or replicated form. Interestingly, a subset of fused cells would present the *"pulverization"* of the DNA belonging to the *interphasic* nucleus: minuscule chromatin fragments would accumulate in the *heterokaryon* suggesting a *genomic catastrophe* and the complete shattering of the interphase cell’s DNA (**figure 47**).

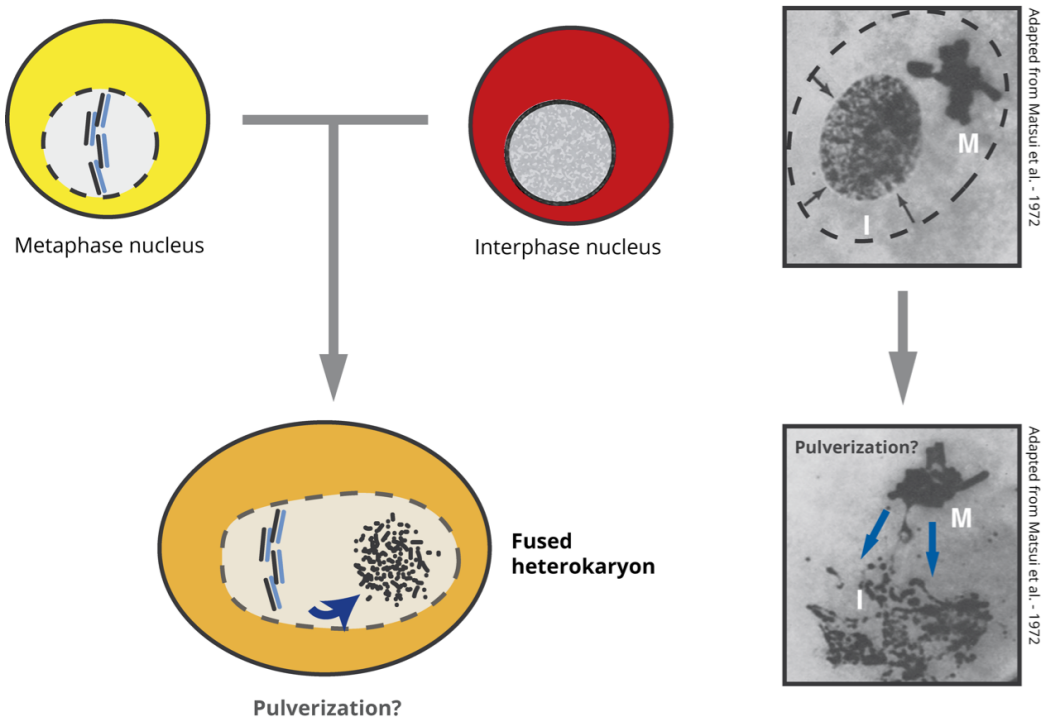



**Figure 47: An unknown agent from a metaphase nucleus can induce the *pulverization* of an interphase cell's genome.** When an *heterokaryon* of a metaphase and an interphase cell is formed, the chromatin of the interphase nucleus condensates and degenerates in a multitude of microscopic fragments, suggesting pulverization.

A tempting interpretation, in agreement with the regulation model proposed for GEN1 and MUS81, would be that the inflow of "licensed nucleases" (or activated cell cycle kinases) provided by the metaphase cell nucleoplasm (in their phosphorylated status), would lead to the pulverization of the DNA undergoing replication.

We decided to test this hypothesis by performing a series of experiments on cells.

By reviewing the literature for this purpose, in search of experimental conditions to replicate the *chromosome shattering* phenomenon, we discovered that cells treated during in S-phase with calyculin A (a potent *Ser/Thr* phosphatase inhibitor) undergo the same "genome pulverization" reported in cell-fusion experiments (Bezrookove, Smits et al. 2003). This would be due to the potent inhibition operated by calyculin A on type 1 and type 2A protein Ser/Thr phosphatases of the PPP family, among which PP1 and PP2A, a negative regulator of PLK1 activity (Lu, Kovach et al. 2009). Calyculin A would thus increase the levels of phosphorylation of mitotic kinases in S-phase.

As a consequence, the persistently activated PLK1 would licence, in a disregulated fashion, the structure specific nucleases, inducing the shattering of replicating chromosomes in the wrong phase of the cell cycle.

We hence tested for conditions that would reproduce the same results reported in the literature (Bezrookove, Smits et al. 2003) (**figure 48**).

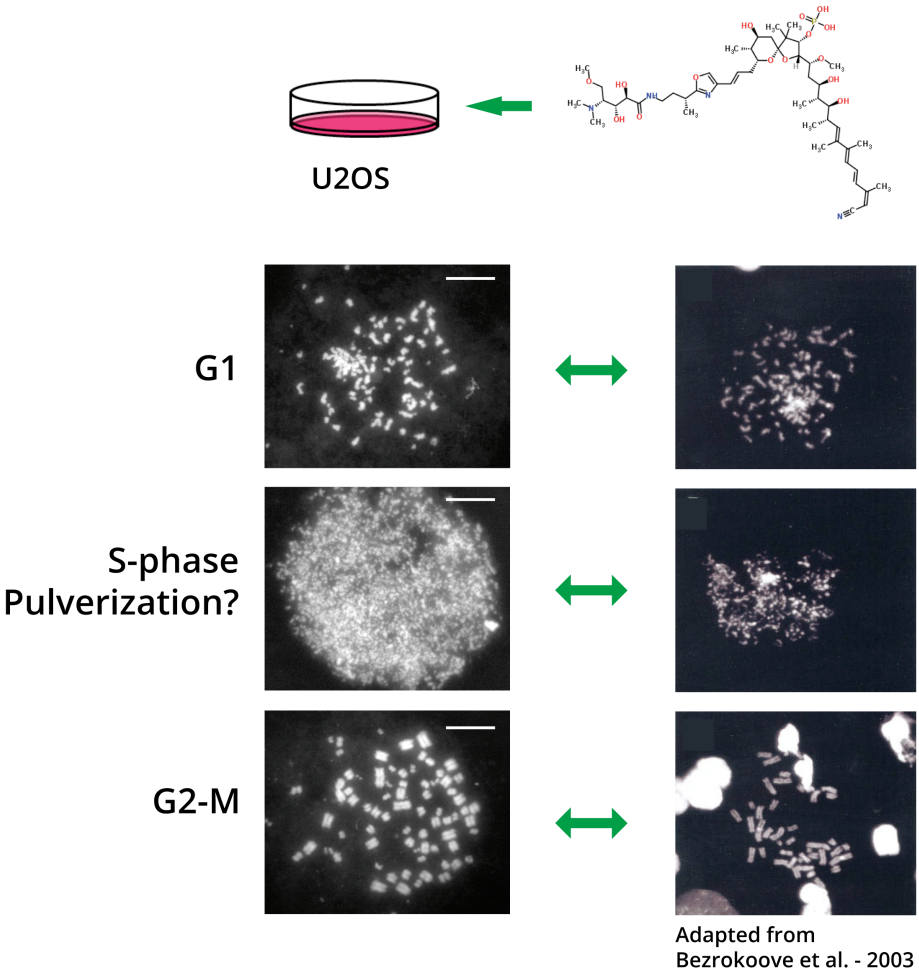



**Figure 48: U2OS undergo chromosome condensation and S-phase pulverization upon treatment with calyculin A.** U2OS cells treated with 80nM calyculin A for 1 hr. replicate the PCC and the S-phase pulverization phenotypes reported (Bezrookove, Smits et al. 2003).

Therefore, we treated with calyculin A U2OS cells or NIH3T3 fibroblasts according to the established conditions.

We thus monitored the accumulation of 53BP1 foci in cells exposed to calyculin to determine whether the conditions generating chromosome pulverization generate actual DNA breaks. (**figure 49**).

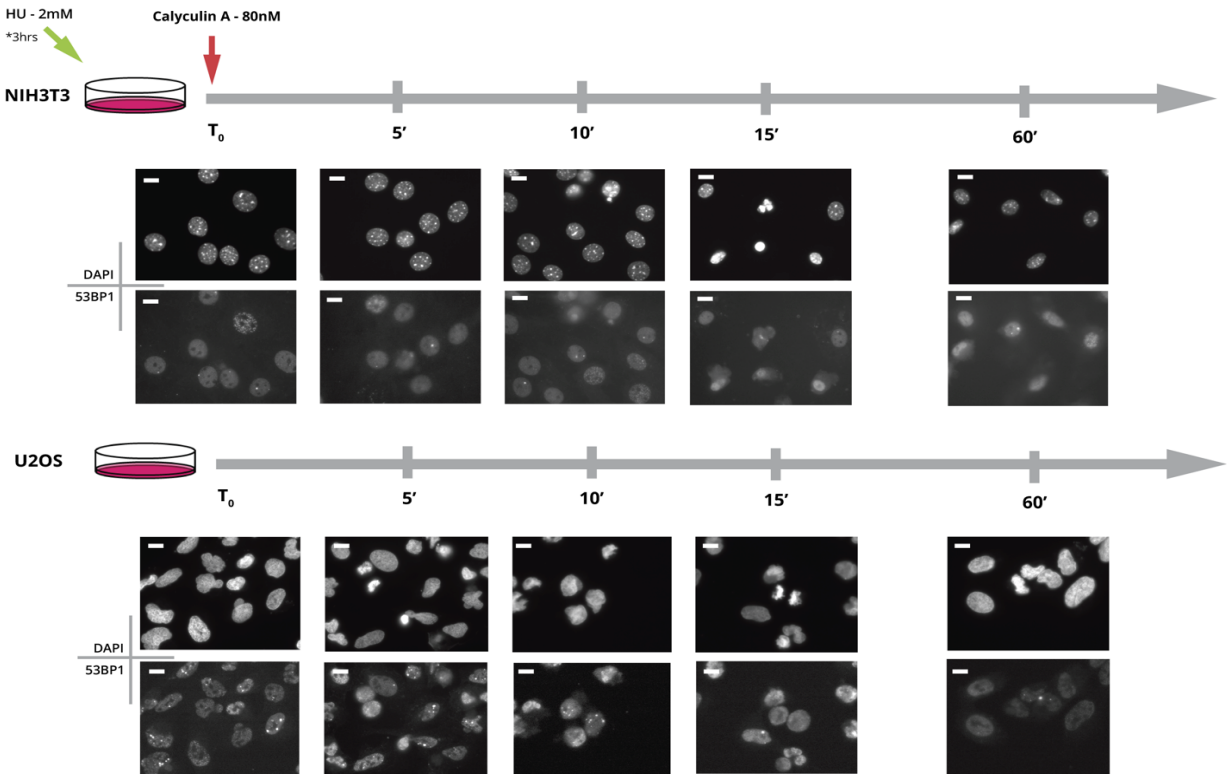



**Figure 49: Treatment of cells with calyculin A doesn't promote the accumulation of 53BP1 foci in cells.** U2OS or NIH3T3 cells treated with a short pulse of 80 nM calyculin don't show accumulation of DNA double strand breaks (indirectly monitored by immunofluorescence against 53BP1).

Since we failed to notice such response, we inferred that the short-term inhibition of PP2A and PP1 wouldn't result in the misregulated activation of structure specific nucleases, at least inside the time interval we chose for the experiment.

The toxicity of calyculin could be accounted for the lack of DSB in treated cells, since we couldn't indeed rule out a negative effect of proliferation of our treatment.

By preventing replication, calyculin would both counteract the generation of DNA replication intermediates in cells and promote, contemporarily, the disregulated

activation of GEN1 and MUS81. As a result, the licensed nucleases would be deprived of their specific DNA substrates and wouldn't hence operate.

An alternative plausible possibility was that the pulverization phenotype we observed in S-phase could be due to the deregulated compaction and condensation of chromatin, rather than to its nucleolytic fragmentation, as historically debated (Bezrookove, Smits et al. 2003). With the aim of testing these speculative possibilities, we arranged for an alternative experimental setup.

U2OS and NIH3T3 were grown in the presence of BrdU, a nucleotide analogue that gets integrated in the nascent DNA strain originating at replication. After the BrdU pulse, cells were treated with calyculin A as in the protocol depicted in **figure 50**.

Additionally, we exposed cells to a PLK1 inhibitor that, according to our speculative hypothesis, would hamper the phosphorylation-mediated licensing of GEN1 and MUS81.

After the treatments, cells were fixed, stained for BrdU and DAPI, and the quantity of nuclei showing a pulverized phenotype was scored.

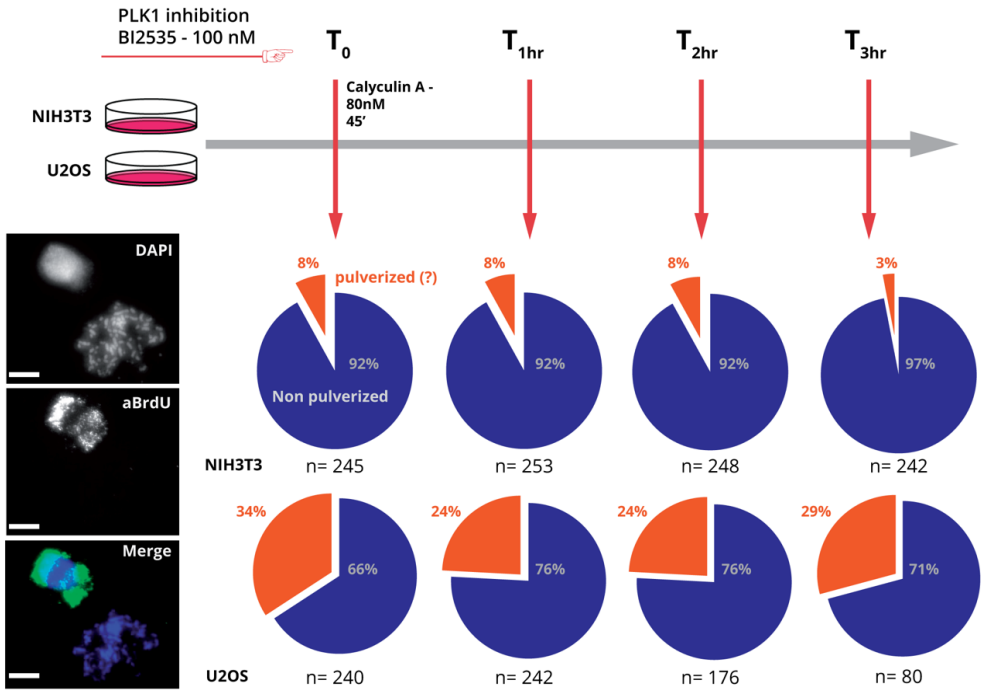



**Figure 50: The inhibition of PLK1 doesn't affect the amount of pulverized nuclei in replicating cells #1.** The amount of cell nuclei showing a pulverized phenotype doesn't correlate significantly with the inhibition of PLK1.

When restricting the scoring to the BrdU⁺ nuclei, we observed that inhibiting PLK1 doesn't have an impact on the amount of pulverized nuclei appearing after calyculin treatment, indirectly supporting the independence of this phenomenon from the phosphorylation status of nucleases (**figure 51**).

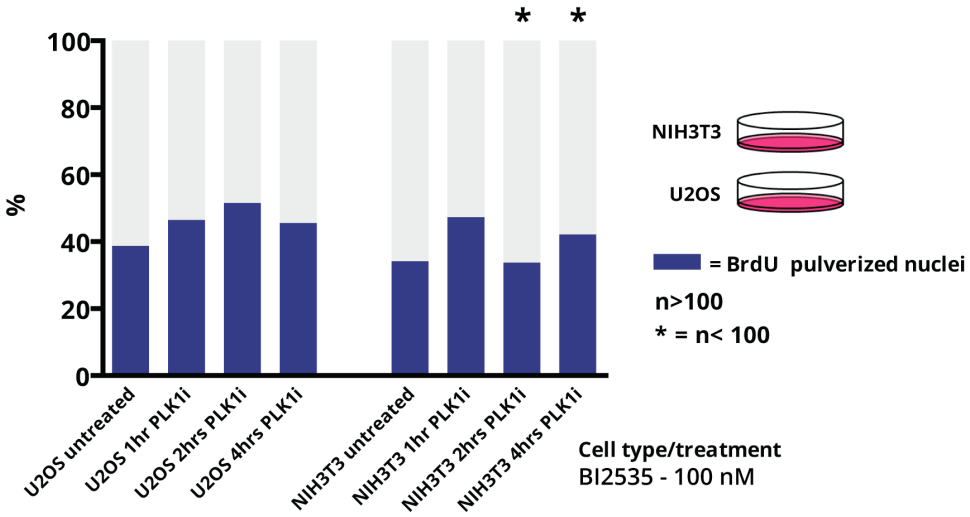



**Figure 51: The inhibition of PLK1 doesn't affect the amount of pulverized nuclei in replicating cells #2.** When restricting our scoring to BrdU⁺ nuclei, we couldn't stress any change in the amount of nuclei undergoing pulverization upon PLK1 inhibition, supporting the independence of what called *chromosome pulverization* from PLK1 activation (hence, from activation of the resolution endonucleases, according to the current model).

Finally, in order to directly evaluate a potential role for the resolution nucleases in chromosome pulverization, we pulsed U2OS cells with BrdU and then treated them with siRNAs against *GEN1* and *MUS81*, verifying the effective downregulation of the genes (**figure 52-B**).

We thus exposed siRNA-treated cells to calyculin and quantified the BrdU⁺ metaphases showing a pulverized phenotype (**figure 52-A**)

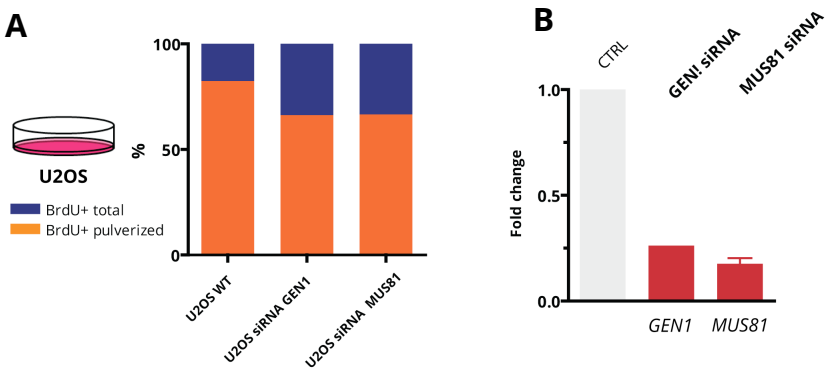



**Figure 52: siRNA mediated down-regulation of *GEN1* and *MUS81* doesn't affect chromosome pulverization.** After silentincing *GEN1* and *MUS81* in U2OS cells (B), we scored the number of pulverized

nuclei after exposing cells to a pulse of BrdU (A). Upon calyculin treatment, we couldn't emphasize differences between siRNA-treated samples and control cells.

The results shown in **figure 51** and **figure 52** suggest that the phenomenon of chromosome pulverization phenotype we observed is not mediated by the resolution endonucleases MUS81 or GEN1, at least in the cellular models we employed.

## Development of a platform to direct at will the activity of resolution nucleases

We finally decided to explore the possibility of generating a cellular system where the activity of GEN1 or MUS81 could be unleashed at will.

The rationale behind this endeavour is directly connected to our genetic exploration on the interplay between dissolution and resolution. Our efforts were following the wealth of literature in yeast defining the genes implicated in resolution as hubs for *synthetic sickness* interactions. Their detrimental interplay with a plethora of genes involved in the prevention of replication stress and genomic instability - among which members of the SMC5/6 complex - suggested the possibility of exploiting their lethal genetic interactions in mammals for clinical purposes. The pharmacological inhibition of resolution nucleases in genetic contexts giving rise to synthetic sickness would represent a novel therapeutic approach along the line with what actively pursued in the context of cancer biology recently (Rehman, Lord et al. 2010).

In order to therefore test for potential inhibitors to use therapeutically, we were in need of appropriate screening tools to monitor and direct the action of nucleases in living cells.

With this purpose in mind, we devised an artificial system aimed to control the activity of nucleases at will. We thus generated a chimeric version of both GEN1 and MUS81 whose intracellular localization could be controlled by the addition of 4-hydroxytamoxifen (4-OHT). We assembled a retroviral system where the coding sequences of both GEN1 and MUS81 were fused to the *tamoxifen-responsive* element of the oestrogen receptor (figure 51-A) and tested our constructs in NIH3T3 cells, verifying the expression of the chimeric proteins and their concentration in the nucleus upon the addition of 4-OHT (**figure 53-B**).

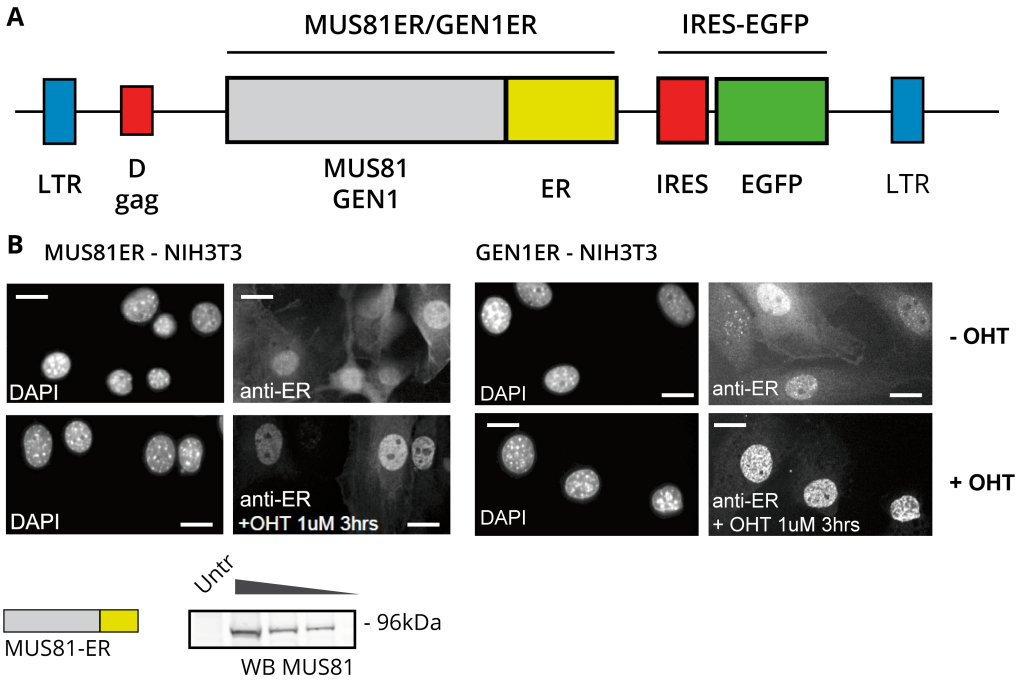



**Figure 53: Chimeric MUS81-ER and GEN1-ER are expressed in cells and translocate to the nucleus upon administration of 4OHT.** NIH3T3 cells were transduced with either MUS81-ER or GEN1-ER (panel A), sorted for top GFP expression and treated with 4-OHT. Upon tamoxifen treatment, both proteins effectively translocate to the nucleus (panel B)

After these initial verifications, we checked whether the re-localization to the nucleus would produce DSBs on the cells' DNA. Therefore, we employed NIH3T3 cells transduced with MUS81-ER, which were sorted for high GFP expression and examined, by high-throughput microscopy, the accumulation of nuclear 53BP1 foci (i.e. the generation of DSBs on chromatin) upon the administration of 4-OHT. We concomitantly treated cells with 2mM hydroxyurea, which we reasoned could increase the amount of nucleases substrates by promoting the generation of DNA joint molecules (**figure 54**).

We indeed hypothesized that by increasing the concentration of DNA replication intermediates, hence favouring the activity of structure specific endonucleases, we should obtain a clearer response from our experimental system.

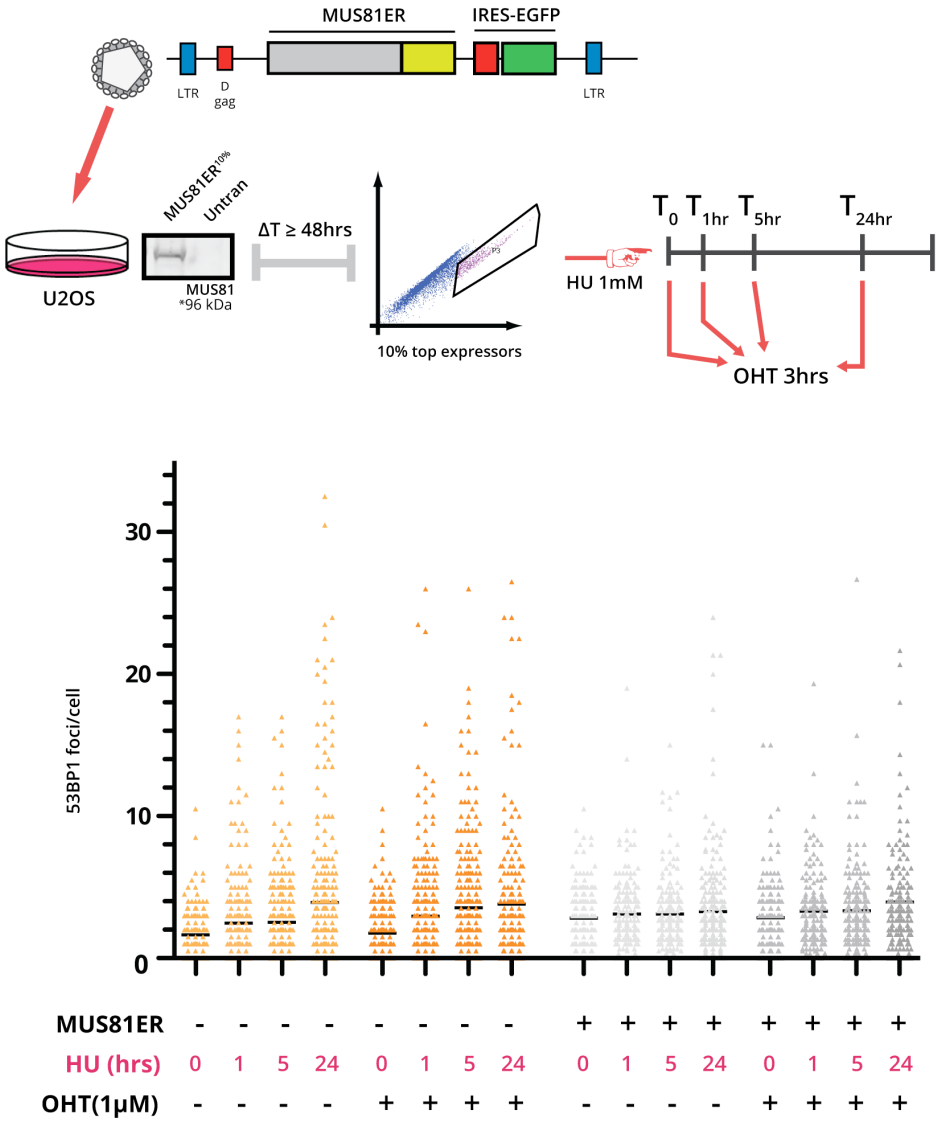



**Figure 54: U2OS$^{MUS81ER}$ cells respond to HU, accumulating 53BP1 independently of MUS81-ER overexpression and localization.** U2OS transduced with MUS81-ER, sorted for GFP expression and treated incrementally with HU show a mild proportional accumulation of 53BP1 foci. Such accumulation though, does not correlate with the nuclear concentration of MUS81.

While a positive trend in the accumulation of nuclear 53BP1 after HU treatment was noticeable, we couldn't see any increase of 53BP1 foci after the administration of 4-OHT to cells.

The same result was confirmed when we repeated this experimental strategy with murine NIH3T3 cells (**figure 55**). As highlighted by the immunofluorescence against 53BP1, no major increase in the quantity of DSBs foci could be noticed after the administration of 4-OHT to cells.

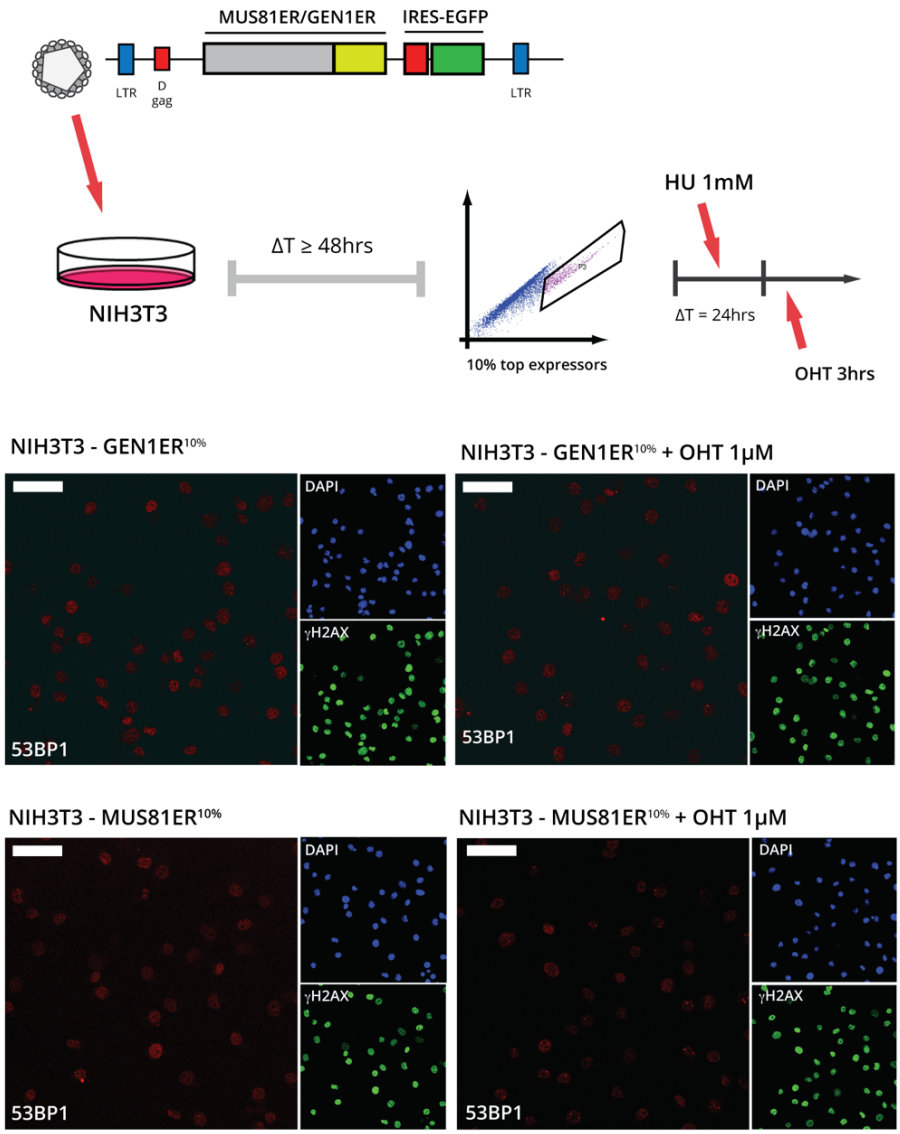



**Figure 55: Nuclear overexpression of GEN1 and MUS81 doesn't boost the processing of replication intermediates in NIH3T3 fibroblasts.** We directly visualized the accumulation of nuclear 53BP1 foci in NIH3T3 cells upon the induction of replication stress through the administration of hydroxyurea. No clear correlation between OHT treatment (i.e. relocalization of endonucleases to the nucleus) and 53BP1 foci could be emphasized. Scale bar: 50μM

In summary, these experiments suggested that the overexpression and relocalization of structure specific nucleases to their site of action is not sufficient for triggering their activity in interphase cells. The lack of a coordination with the endogenous regulation of these enzymes might account for such lack of feedback. Additionally, the nuclear saturation resulting from the ER-driven localization might interfere with the correct processing of DNA replication intermediates, hampering the any functional response.

Furthermore, the steric hindrance of the ER moiety we fused to the sequence of MUS81 and GEN1 could represent an additional technical drawback. The presence of an exogenous protein sequence attached to the protein backbone may result in the alteration of the enzymatic activity of the "chimeric" nucleases, hence frustrating the possibility of employing them to process DNA replication intermediates at will.

In summary, several optimizations could be applied in order to improve the synthetic platform we envisioned and tentatively tested.

We are indeed convinced of the validity of our approach, and we’re confident that further developments may lead to an optimized platform to harness, on the long term, the therapeutic potential embraced by the metabolism of joint DNA molecules

# Discussion

# Discussion

## Part I: Framing the role of SMC5/6 in metazoans: the need for experimental tools.

Our study represents a tentative approach towards a comprehensive characterization of the SMC5/6 complex in metazoans. The very lack of a common name recalling its function is, by itself, quite a telling feature, differentiating the complex from its closest partners, cohesin and condensin. As previously mentioned, the SMC5/6 dimer is essential both in lower and higher eukaryotes (Ju, Wing et al. 2013; Lehmann, Walicka et al. 1995), and this has historically represented a major drawback, discouraging any genetic endeavours aimed at defining its function in metazoans.

As a demonstration of our knowledge gap, at today's date, most literature on the function of the human SMC5/6 dimer derives from studies based on the siRNA depletion of its individual components (Potts and Yu 2005; Potts, Porteus et al. 2006, Potts and Yu 2007; Behlke-Steinert, Touat-Todeschini et al. 2009). Only recently a first tentative approach of gene targeting , performed in chicken DT40 cells (Stephan, Kliszczak et al. 2011), has provided some surprising data on the essentiality of the complex in metazoans.

We explored this scenario along the line traced by yeast literature and develop mouse models recapitulating the SMC5/6 phenotypes described in lower eukaryotes.

### *Nsmce2* is an essential gene in higher eukaryotes, while its SUMO ligase activity is dispensable.

The first endeavour of the lab - a KO mouse for *Nsmce2* - yielded a perfect correlation with the yeast data. As observed in both *S. cerevisiae* and *S. pombe* (Giaever, Chu et al. 2002; Kim, Hayles et al. 2010), *Nsmce2*$^{KO}$ mice are unviable, aborting development at the blastocyst stage (Jacome et al. unpublished).

The next approach undertaken - a functional impairment of NSMCE2, rather than its systemic deletion - represented a successful compromise to overcome the lethality observed in the *Nsmce2*$^{KO}$ mice.

The strategy to abrogate the SUMO ligase activity of NSMCE2 was corroborated experimentally, since we could verify that the SP-RING mis-sense C185S;H187A

mutations, originally described and featured in *S. pombe* (Andrews, Palecek et al. 2005), equally affected the murine protein and severely impaired its activity towards SUMOs.

Despite a mild instability phenotype highlighted in cells, mice bearing the mutation didn't show altered fitness, and their overall health and survival was unaffected. These data well agree with many reports on SUMO biology in Vertebrates. Whilst SUMOylation is essential for viability in *S. cerevisiae* (Giaever, Chu et al. 2002) , in metazoans its function is at least partially dispensable (Zhang, Mikkonen et al. 2008)

**NSMCE2: a bona fide SUMO2 ligase in higher eukaryotes.**

When characterizing biochemically NSMCE2$^{SD}$, we could highlight its SUMO2-ligase *loss of function*, with only a partial effect on SUMO1 conjugation, suggesting that NSMCE2 is a *bona fide* "SUMO2-preferring" ligase in higher eukaryotes. Even though we could pinpoint a functional bias towards SUMO2, we cannot rule out the possibility of a SUMO1 conjugating activity of the protein *in vivo*, considering that NSMCE2 has been described as a SUMO1 ligase in alternative biological systems (Potts and Yu 2005).

It is important to remark how the specificity towards SUMO paralogues exerted by the handful E3 ligases encoded in metazoans is still rather uncharacterized. A certain "isoform versatility" is often observed when performing SUMOylation experiments *in vitro*, as in the case of enzymes with a defined isoform preference *in vivo* (Vertegaal, Andersen et al. 2006).

**NSMCE2$^{SD}$ cells as a platform to define the NSMCE2 SUMOylome**

The *Nsmce2$^{SD}$* model we developed represented a tentative instrument to characterize of the NSMCE2 SUMOylome *in vivo*.

As previously mentioned, only few NSMCE2 targets have been described in higher eukaryotes - among them SMC6, SMC5, NSMCE2 itself and some members of the *shelterin* complex at telomeres (Potts and Yu 2007). Most data in this context originates from *in vitro* conjugation experiments, an approach bearing intrinsic flaws that we wanted to overcome.

We designed for the purpose different tagged versions of SUMOs to specifically identify the NSMCE2-modified fraction of the proteome *in vivo*, and took advantage of *SUMO-dead* immortalized fibroblasts as a *semi-endogenous* expression platform. We reasoned that, by

comparing purified lysates from wild-type cells and *SUMO-dead* counterparts by mass spectrometry, we should come across a pattern of SUMOylated proteins that would not be represented in the *NSMCE2*$^{SD}$ purified sample. Their exclusive presence in the wild-type pool would hence signify that NSMCE2 is implicated in their SUMOylation.

After embarking in such endeavour, we soon encountered a variety of major downsides. As previously mentioned, murine fibroblasts are often refractory to exogenous protein expression, and despite the possibility of enriching for the top expressing cell populations, we constantly obtained very low yield of SUMO-overexpression in our samples.

Additionally, top expressors commonly responded to the high load of SUMOs either arresting proliferation or quenching the expression of the construct transduced (probably by epigenetic silencing). Whenever sufficient amounts of overexpression could be obtained (allowing for the detection of free SUMO in cells by Western blot), we routinely tested for the presence of SUMO-modified SMC5-SMC6 or NSMCE2 as experimental validation, failing to detect any of these described targets.

A possible explanation may be linked to the intrinsic lability of SUMOylation as a post-translational modification. Additionally to its transitory nature (Geiss-Friedlander and Melchior 2007), the purification and subsequent detection of SUMO-modified proteins can be hampered by their paucity in pooled samples.

In summary, we esteem that a variety of synergistic factors frustrated our quest for the endogenous targets of NSMCE2: a poor overexpression platform, the relative scarcity of NSMCE2 targets in the proteome, and the poor sensitivity of our validation system.

As outlined previously, there would be several ways to boost our approach. As an example, we could take advantage of recent optimized pipelines for the purification of endogenously SUMOylated targets (Becker, Barysch et al. 2013). Alternatively, we could focus on endogenous SUMOylation by specifically labelling SUMO genes through genome editing, avoiding thus the issues provoked by overexpression of exogenous SUMO in cells.

### *Nsmce2*$^{SD}$ cells show an instability signature with no major consequences at the organism level

An interesting feature of our *Nsmce2*$^{SD}$ model is the recapitulation, at least at the cellular level, of the instability phenotype observed in yeast *mms21* and *sgs1/Blm* mutants. Similarly to the yeast case, we could verify that the impairment of the SUMO ligase activity of NSMCE2 brings about an increased sensitivity towards replication stress and the associated DNA damage *in vitro* (Andrews, Palecek et al. 2005). We reckoned such similarity by quantifying the nuclear by-products of aberrant DNA segregation in replicating cells - micronucleation and polynucleation - which are routinely employed as readout of genomic instability in cells (Fenech, Kirsch-Volders et al. 2011). While *Nsmce2*$^{SD}$ cells behaved according to our expectations, based on the accumulated evidence in lower eukaryotes, we were surprised by the lack of a reflected phenotype *in vivo.*

Even though no changes in the survival rates of *SUMO-dead* mice could be highlighted, yet the animals showed a tendency to spontaneous colon tumour formation, as underlined by their worse response to induced tumorigenesis, again recapitulating an hallmark of the *Bloom* syndrome.

### Combining *Nsmce2*$^{SD}$ with *Mus81*$^{KO}$ doesn't affect fitness.

These observations, together with the mild instability signature observed at the tissue level, led us to employ the *SUMO-dead* strain as a first *chassis* for genetic permutations with the resolution machinery. In our expectations, the negative effects of the *Nsmce2*$^{SD}$ allele should be exacerbated by the impairment of resolution, attained by combining the strain with *Mus81*$^{KO}$ animals. Again unexpectedly, that didn't prove to be the case - at least for the exploratory *Nsmce2*$^{SD}$*: Mus81*$^{KO}$ cross generated. The *synthetic sickness* we hypothesized was falsified by the lack of major cellular or systemic phenotypes in double mutant animals.

### Resiliency and robustness characterize the processing of DNA joint molecules

The most straightforward interpretation of our data lies in the robustness and resiliency of pathways such as dissolution and resolution, where several enzymes play a concomitant role. Though MUS81 seems to have a preeminent role in avoiding the accumulation of DNA joint molecules (Ying, Minocherhomji et al. 2013; Naim, Wilhelm et

al. 2013), the compensating effects of GEN1 and SLX4-SLX1 upon its deletion can't obviously be ruled out *a priori*.

Our efforts after the shRNA-mediated depletion of *Gen1* **and** *Slx4* in *Nsmce2$^{SD}$: Mus81$^{KO}$* MEFs further supported this interpretation. The experimental difficulties we encountered, together with the lack of specific effects, was suggesting the need for a genetic deletion, rather than a simple depletion, of these fundamental *inter-players*.

### *Smc6$^{S994A\text{-}neo}$* as an alternative *genetic chassis* to define the SMC5/6 – resolution interplay

In order to gain further insights and to overcome the lack of a well-defined phenotype encountered with the *Nsmce2$^{SD}$* mice, we opted for an alternative genetic carrier. The recently published hypomorphic *Smc6$^{S994A\text{-}neo}$* model (Ju, Wing et al. 2013) responded to our need for a functional mutation of SMC5/6 that would bear a marked and quantifiable phenotype. Intriguingly, the *Smc6$^{S994A\text{-}neo}$* - *Mus81$^{KO}$* strain showed rather dramatic *synthetic lethality*, which frustrated our ability to obtain both experimental animals and cells.

While on one side this strongly supported our starting hypothesis of a functional interplay SMC5/6 - resolution, it also suggested that, intriguingly, NSMCE2 and SMC6 could possibly possess a similar contextual role (the prevention of DNA replication intermediates) exerted through different molecular pathways, opening the possibility of further genetic dissection.

The additional finding of the genetic linkage between *Gen1* and *Smc6* called our attention. As mentioned, it might represent the result of a tight interdependence of the two genes, which would undergo selective pressure to transmit across generations as a single functional unit, as in the case of many non coding DNA elements (such as miRNAs) (Altuvia, Landgraf et al. 2005).

### *Smc6$^{S994A\text{-}neo}$* is synthetic lethal with *Mus81$^{KO}$* - the need for an alternative platform

The scenario illustrated by the *Smc6$^{S994A\text{-}neo}$Mus81$^{KO}$* intercross, which somehow complemented the lack of phenotypes of *Nsmce2$^{SD}$-Mus81$^{KO}$* animals, was the reason to translate our genetics towards a novel carrier - a conditional strain for *Nsmce2* that

recapitulated extensively *Blm/Sgs1* phenotypes, recently developed in the lab (Jacome et al. unpublished).

In order to reduce the experimental noise represented by the action of GEN1 and SLX1 in the absence of MUS81, and to highlight potential phenotypes that may be hidden by this redundancy, we proceeded to generate concomitantly a conditional *Nsmce2$^{lox}$ - Mus81$^{KO}$* and a *Nsmce2$^{lox}$-Gen1$^{KO}$-Slx4$^{lox}$* strain.

The latter represented the optimal experimental tool for our studies since it allowed to overcome genetically the issues encountered employing shRNA procedures to depleting structure specific nucleases. As previously mentioned, moreover, targeting *Slx4* would contemporarily impair the function of MUS81 and SLX1, taking into account that SLX4 has been described as a coordinating platform for the activity of these two enzymes (Fekairi, Scaglione et al. 2009).

In addition to its *bifaceted* effect, the *rationale* for deleting *Slx4* instead of directly involving MUS81 was due to the synthetic lethality reported for *Gen1*$^{KO}$: *Mus81*$^{KO}$ mice (Matthews et al. unpublished data).

The choice of a conditional allele for *Slx4* and *Nsmce2* was due to the lethality observed both in our *Nsmce2$^{KO}$* mouse model and in the *Slx4$^{KO}$* strain (Castor, Nair et al. 2013). It allowed us, moreover, to specify the working cell model, overcoming the experimental flaws of fibroblast, a cell type which often arrested replication after experimental manipulation, hampering the possibility of stressing any replication-related phenotype related to the underlying genetics.

We hence generated both a by *Nsmce2$^{lox}$ - Mus81$^{KO}$ CD19Cre and a Nsmce2$^{lox}$-Gen1$^{KO}$-Slx4$^{lox}$ CD19Cre* to narrow our analysis on B-lymphocytes, a versatile system where we could take advantage of a genetically-encoded Cre.

**MUS81 processes the recombination intermediates leading to SCEs in *Nsmce2$^{\Delta/\Delta}$ cells.***

Interestingly, we could and confirm a reported phenotype associated with the concomitant ablation of *Blm* and the silencing of *MUS81* in human cells (Wechsler, Newman et al. 2011). While *Nsmce2$^{\Delta/\Delta}$* primary fibroblasts undergo SCEs with a frequency comparable to that of *Blm* cells, the absence of MUS81 reverts the phenotype to control

levels, suggesting the specific implication of MUS81 in the processing of recombination intermediates arising from the impairment of SMC5/6 Our additional observations in *Nsmce2$^{lox}$ - Mus81$^{KO}$ CD19Cre* reinvigorated the similar observations preliminarily obtained in MEFs.

Though we could indeed reconfirm the SCE reversion phenotype previously outlined, surprisingly, no additional cellular parameters resulted being affected.

While this unexpected result may be related to the cell specific effects, it also corroborated the intriguing possibility of a separation of function between *Nsmce2* and the core SMC5/6 complex in their interplay with *Mus81*. The dramatic lethality of the *Smc6*/*Mus81* genetic combination we analyzed could in fact result from the impairment of a yet-to-define regulatory mechanism operated by the SMC5 core complex independently from the function of *Nsmce2*.

***Nsmce2$^{\Delta/\Delta}$: Mus81$^{KO}$* MEFs don't show signs of synthetic sickness.**

Apart from the interesting observation on the SCE rates, the preliminary analysis we performed on *Nsmce2$^{\Delta/\Delta}$-Mus81$^{KO}$* MEFs failed to stress any signature of synthetic sickness.

Again, due to a probable redundancy of function between MUS81 and the alternative resolution nucleases, the overall "well-being" of cells didn't result affected negatively by the underlying genetics. We did notice once more, instead, the detrimental effects of the experimental manipulation mentioned above. The adenoviral transduction of Cre, as well as the retrovirus-mediated transduction of SV40$^{T121}$ profoundly affected several of the parameters we assayed - notably, cell proliferation and the rates of micronucleation and polynucleation.

**The SMC5/6 complex: coordinating dissolution?**

In conclusion, our genetic findings generally supported the hypothesis of a functional relationship between the SMC5/6 complex and resolution in metazoans, framing its role in the context of dissolution, similarly to the case of *S. cerevisiae* and *pombe* (Bermudez-Lopez, Ceschia et al. 2010, Branzei, Sollier et al. 2006).

The phenotypes of the *Nsmce2* mutants we involved in our study recapitulated what observed in yeast (at least at the cellular level, in the case of the *Nsmce2$^{SD}$* allele). Whilst

we failed to highlight a strong *in vivo* phenotype when combining *Nsmce2* mutations with *Mus81* deletion, we could verify that affecting directly the core of the SMC5/6 complex (the *Smc6*$^{S994A\text{-}neo}$ allele) on a *Mus81-null* background results in synthetic lethality (**figure 56-A**).

Such a drastic phenotype, standing somehow at opposite extreme of what observed when distressing the function of NSMCE2, strongly suggested that the SMC5/6 shouldn't be considered as a single functional unit, but rather that it could operate as a regulatory platform coordinating different pathways implicated in the processing of joint DNA molecules, as depicted in **figure 56-B**.

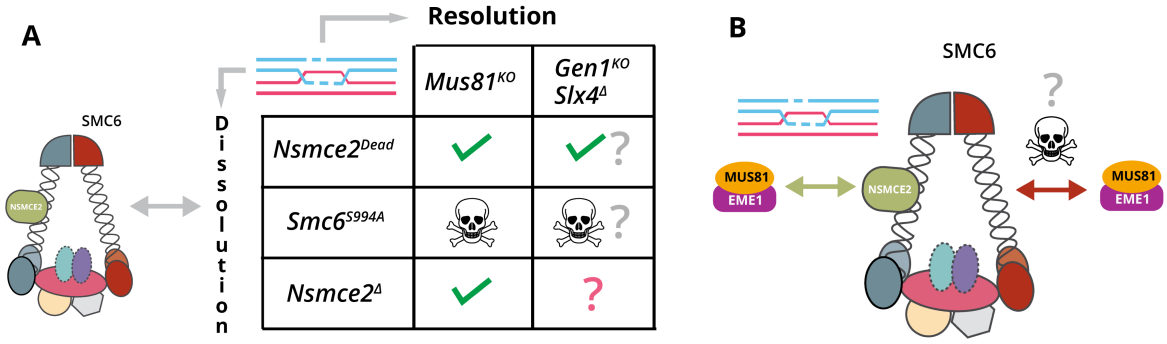



**Figure 56: Genetic permutation between the SMC5/6 complex and the resolution machinery.** Our study suggest the implication of the SMC5/6 complex in the processing of joint DNA molecules in higher eukaryotes. While the concomitant impairment of *Nsmce2* and *Mus81* produces minor detrimental consequences, perturbing the core complex in a *Mus81*$^{KO}$ background results in synthetic lethality (panel A). These contrasting outcomes support the idea of the SMC5/6 as a coordinating interface for different molecular pathways (panel B)

Further genetic dissection of the specific function of different members of the SMC complex could probably shed a light on the reason for the synthetic lethality we observed, as well as for the mild phenotype generated by altering the function of NSMCE2 alone.

### The transition from replication to cytokinesis and the trafficking of DNA joint molecules through dissolution and resolution

In our speculative view, the combined impairment of dissolution and resolution would result in an anomalous accumulation of DNA joint molecules after replication. The "traffic jam" resulting would promote the formation of anaphase bridges at cytokinesis,

resulting finally in unbalanced chromosome segregation and additional genomic injuries that could further degenerate into LOH and, potentially, cancer.

Our findings supported a scenario like the one depicted in **figure 57**.

By concomitantly mutating NSMCE2 and impairing of the resolution machinery (*knocking-out* MUS81), we compromised the processing of joint DNA molecules and verified, at least at the cellular level, the instability features associated.

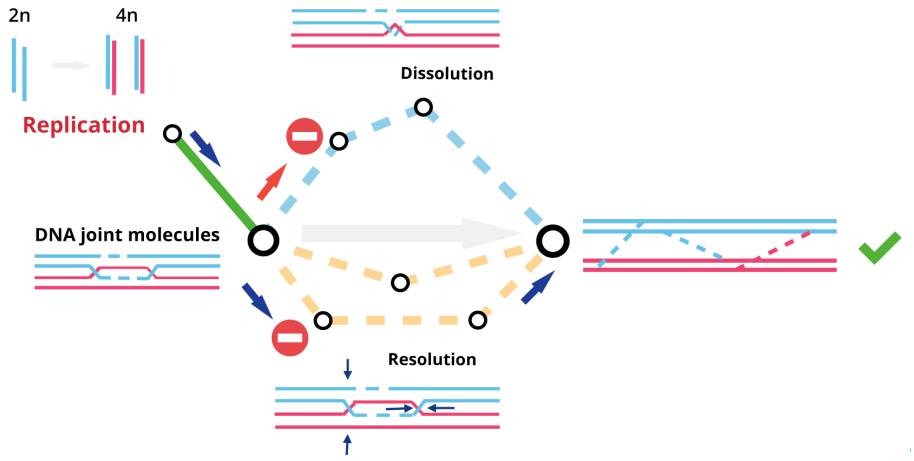



**Figure 57: hampering dissolution and resolution through mutations affecting NSMCE2 and MUS81 resulted in mild instability *in vivo*.** The lack of a strong phenotype after the impairment of the processing of replication intermediates probably result from the intrinsic resiliency of redundant systems to perturbation.

### SMC5/6 mutants phenocopy *Blm* mutations in higher eukaryotes, but the complex may operate on different pathways.

It's important to stress again how our strategy to impair dissolution relied on mutating functionally some of the proteins of the SMC5/6 complex – namely, *Nsmce2* and *Smc6* – taking into account the justified assumption of a *genetic synonymy* between *Smc5/6* and *Blm*. One possibility to explain the lack of major consequences in the case of the *Nsmce2/Mus81* genetic combination might imply the functional up-regulation of dissolution, through an up-regulation of *Blm,* as in **figure 58**.

Alternatively, NSMCE2 could operate in an undefined regulatory mechanism whose alteration might results phenotypically in dysfunctional dissolution, as outlined in **figure 59**.

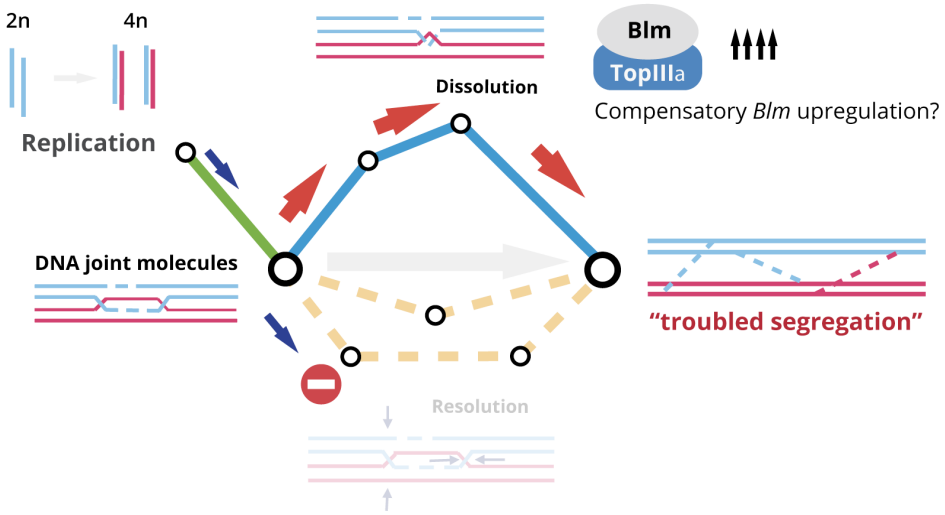



**Figure 58: *Blm* up-regulation could compensate for the impairment of the SMC5/6 complex.** A possible explanation to the lack of strong phenotypes observed when mutating NSMCE2 and deleting MUS81 could be provided by the up-regulation of *Blm*, the direct mediator of dissolution.

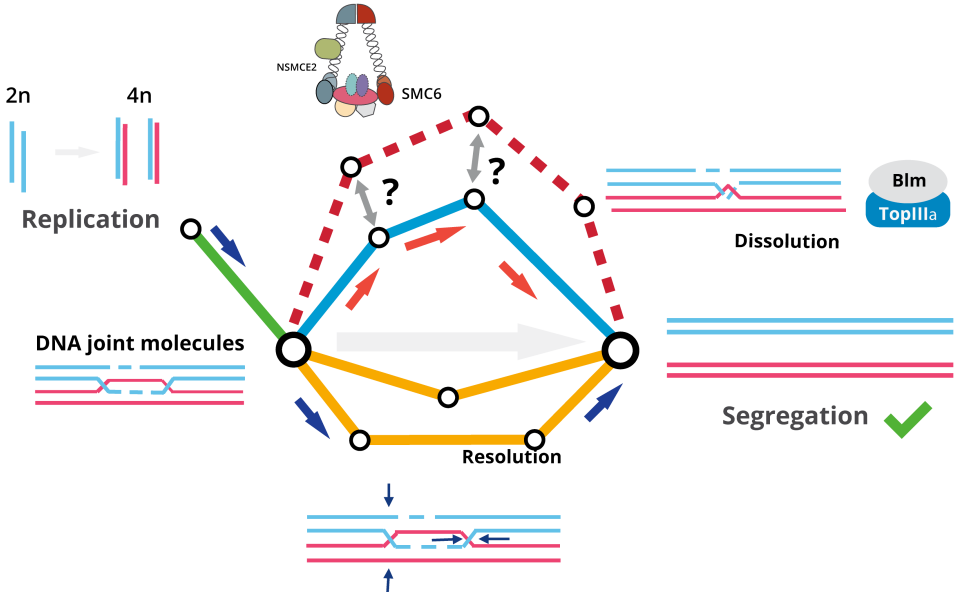



**Figure 59: the SMC5/6 complex could mediate a regulatory mechanism interplaying with resolution.** Mutations in either the core SMC5/6 complex or in associated partners (such as NSMCE2) might result in "dissolution phenotypes", despite being unrelated from the actual processing of DNA joint molecules.

In summary, rather than having a unique role in the metabolism of DNA replication intermediates, the SMC5/6 complex could be implicated as a regulatory hub for different pathways whose alteration could have a resulting effect on dissolution. As we were able to show, different functional members of the complex could exert alternative roles along uncharacterized pathways and, as a consequence, the phenotypes associated to their mutation may be reflected to different extents in dissolution.

Further genetic analysis might provide additional evidence for this possibility, as it would the global abrogation of resolution in a SMC5/6 mutated background. Experiments on the *Nsmce2$^{lox}$-Gen1$^{KO}$-Slx4$^{lox}$-CD19Cre* strain we're currently generating in the lab should indeed help to clarify this alternative scenario. Along this line recent work in the literature provided the first evidence for the consequences of abrogating nucleases in cells that are *non proficient* for dissolution (Wechsler, Newman et al. 2011). The authors showed how the depletion of GEN1 and MUS81 in a *Blm* deficient background results in a varied array of chromosomal aberrancies (**figure 60-A**), among which the generation of segmented chromosomes (**figure 60-B**), structures that are still poorly understood and so far only observed in transformed cell lines.

We're particularly interested in seeing whether segmented chromosomes should emerge from experiments in our *Nsmce2$^{lox}$-Gen1$^{KO}$-Slx4$^{lox}$* model, further supporting our view of the SMC5/6 as a regulator of the metabolism of DNA replication intermediates.

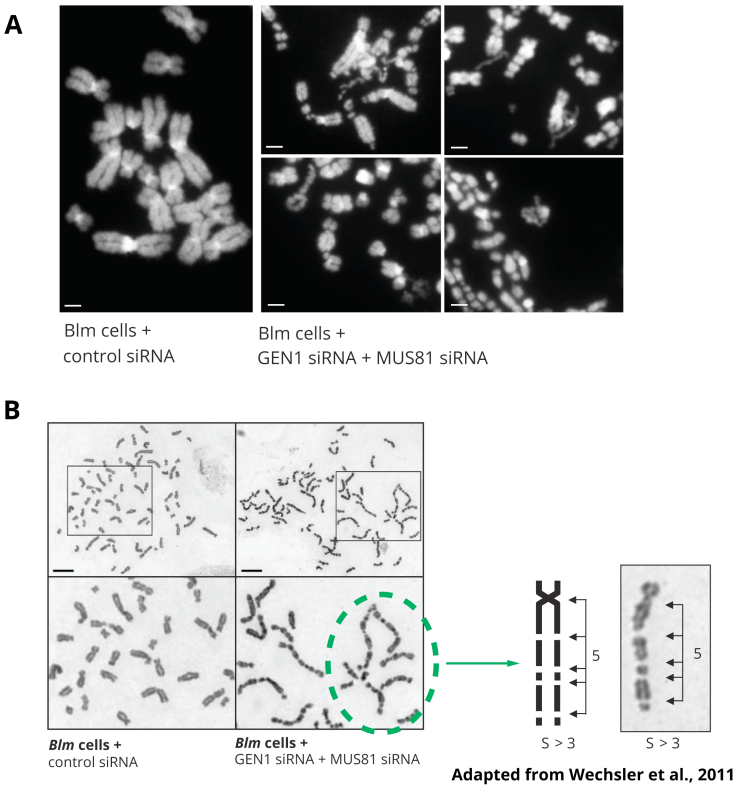



**Figure 60: impairing GEN1 and MUS81 in Blm cells generates chromosomal aberrancies.** When structure specific nucleases are depleted in cells un-proficient for dissolution, chromosomes undergo extensive damage (panel A) and present a segmented morphology (panel B).

## Part II - Studies on the nucleases involved in the resolution pathway

Our efforts in the context of nucleases biology were originally intended as an integrating and complementary side of the main genetic project.

If on one side we planned to unravel the *genomic housekeeping* role of the SMC5/6 complex through genetics, on the other we aimed at potential therapeutic strategies that could be supported by our genetic findings.

### Chromosome pulverization: chromatin fragmentation, deregulated condensation or both?

We first wanted to assay the potential implication of GEN1 and MUS81 in the phenomenon of chromosome pulverization (CP), inspired by the idea that the deregulated activity of endonucleases, triggered in the inappropriate part of the cell cycle, could wreak havoc on genomes.

The pulverization of chromosomes has been at the centre of a long dispute. Two opposite interpretations have for long described it as either a general fragmentation of chromatin or as a partial and deregulated condensation of chromatin. Though a final consensual agreement on the “condensational theory” seems to have established during time, the possible coexistence of both phenomena behind the same phenotypical outcome has yet to be falsified, experimentally (Stevens, Abdallah et al. 2010).

With our trials, we could show that both the transient silencing of *GEN1* and *MUS81* in cells, as well as the inhibition of PLK1, the regulatory kinase proposed as a major regulator of resolution (Matos, Blanco et al. 2011), don't counteract the appearance of chromosome pulverization in replicating cells.

These results could suggest that the levels of PLK1 inhibition and *GEN1*/*MUS81* silencing attained in our experiments were insufficient to inhibit CP. An alternative interpretation could though indicate that CP probably does not depend on the nuclease activity of GEN1 and MUS81, in agreement with the literature supporting the “condensational theory”.

### Designing a system to activate structure-specific nucleases at will

After these descriptive experiments, we thought to operate creatively, applying in the context of resolution the principles of synthetic biology. We thus explored the possibility

of redirecting the action of nucleases at will by designing the ER-fused versions of GEN1 and MUS81. Our goal was to establish a platform to discover nuclease inhibitors allowing for novel therapeutic approaches, based on the synthetic sickness interaction of these genes with several DNA repair mediators.

We thus cloned an ER-endowed version of both proteins to effectively control their cellular localization by administering *4-hydroxytamoxifen*. We hypothesized that, by increasing the nuclear concentration of the nucleases, we should produce an increase in the DSBs that these enzymes operate onto joint DNA replication molecules, especially after challenging replication and producing and accumulation of DNA replication intermediates.

Despite several efforts in different cellular models, we failed to emphasize a direct correlation between the double strands breaks generated on cells and the concentration of either GEN1 or MUS81 in the cellular nucleus.

As previously mentioned, several phenomena may account for this lack of a real function of our platform in living cells. A major drawback could be the represented by the ER fusion strategy we employed, which involves the fusion of the protein of choice with an extended protein moiety from an exogenous protein. In the case of MUS81 and GEN1, the important "steric hindrance" of the ER fragment could be affecting the processing of DNA structures by the nucleases, hampering the generation of a response. Additional work should plausibly be done on the integration of our artificial operators with the endogenous regulation machinery operating on dissolution. We can’t indeed rule out that our artificial nucleases did not work as expected for a lack of an appropriate phosphorylation licensing. Optimizations such as the use of phosphomimetic versions of the enzymes that may override the endogenous regulation could potentially result in a functional system

We are strongly convinced that this approach should be of great help in developing therapeutic strategies targeting the accumulation of joint DNA molecules in the future.

# Conclusions

## Conclusions

### Part I

- The *Nsmce2*$^{SD}$ mutant allele developed in our laboratory severely compromises NSMCE2-dependent SUMOylation activity.
- In agreement with yeast data, *Nsmce2*$^{SD}$ animals showed features in vitro which have been linked to problems on the "dissolution" of joint DNA molecules, including micronuclei or increased levels of recombination.
- In contrast, *Nsmce2*$^{SD}$ animals are born at Mendelian ratios, are fertile and show a normal lifespan that demonstrates how NSMCE2-dependent SUMOylation is largely dispensable for fitness.
- An SMC6 mutant allele is synthetic lethal with MUS81 deficiency in mice, illustrating that mutations compromising dissolution and resolution activities lead to severe consequences.
- In contrast, no effect of MUS81 deficiency is detectable on *Nsmce2*$^{SD}$ mice, reinforcing the lack of a strong phenotype on these animals.
- Deletion of MUS81 reverts the increased recombination rates observed on NSMCE2 deficient cells, suggesting that MUS81-mediated resolution takes care of the joint molecules that cannot be dealt by the SMC5/6 complex.
- Surprisingly, no obvious defect on the overall fitness is observed on NSMCE2 deficient B cells or MEF upon deletion of MUS81. Given the effects observed with the SMC6 mutant allele, it remains possible that more pronounced effects could be observed in other cell types, particularly during embryonic development.

### Part II

- The phenomenon of chromosome pulverization does not depend on the activity of PLK1 kinase or MUS81 and GEN1 nucleases-
- Translocating MUS81 or GEN1 nucleases into the nucleus of S phase cells is not sufficient to trigger their activity, suggesting that additional layers of regulation might restrict their activity to mitotic cells.

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